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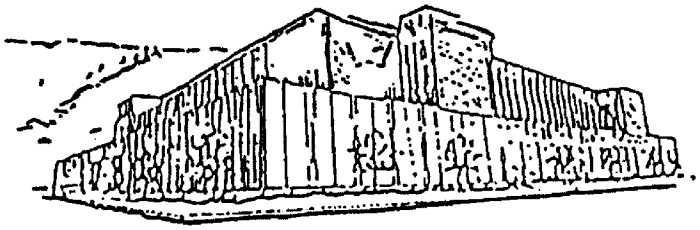

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# The Search for Sex-Linked Markers in Pink Salmon (*Oncorhynchus gorbuscha*)

by

Kristine Laurel Pilgrim

B.A., The University of Montana, 1996

presented in partial fulfillment of the requirements

for the degree of Master of Science

The University of Montana

1999

Approved by:

Dr. Fred Allendorf, committee chair

Dean, Graduate School

5-28-99

Date

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Pilgrim, Kristine L., M.S., May 1999 Organismal Biology and Ecology

**Searching for Sex-Linked Markers in Pink Salmon (*Oncorhynchus gorbuscha*)**

Committee Chair: Dr. Fred W. Allendorf FWA

The goal of this thesis research was to detect and describe sex-linked genetic markers in pink salmon (*Oncorhynchus gorbuscha*). This study was part of an ongoing project to map the pink salmon genome in order to assess possible genetic changes resulting from the March 1989 *Exxon Valdez* oil spill. The first chapter provides a summary of the process of sex chromosome evolution, and a discussion of what is known about sex-linked markers in salmonids.

The second chapter discusses the detection and investigation of a pseudogene derived from growth hormone 2 that is perfectly linked to the sex determining region on the Y-chromosome. This pseudogene (*GH-2p*) provides an unambiguous, PCR-based test for sex and is an important sex-linked marker for pink salmon. I amplified and sequenced the third intron (intron C) from growth hormone 2 (*GH-2*), and found a length polymorphism that is caused by an 81 bp insertion homologous to the 3' end of the salmonid short interspersed nuclear element (SINE) *Sma*I. I performed phylogenetic analysis of intron C of *GH-2p* and *GH-2* from pink salmon and other salmonids and found that the duplication event of *GH-2* that gave rise to *GH-2p* occurred after the divergence of *Salmo* and *Oncorhynchus* but before the divergence of rainbow and cutthroat trout from the other *Oncorhynchus* species.

In contrast to *GH-2p*, which is perfectly linked to the sex determining region on the Y-chromosome, the goal of the research presented in chapter 3 was to detect a sex-linked marker present on both the X and Y chromosomes. Such a marker would allow identification of the X-chromosome linkage group. I used bulked segregant analysis and screened 74 amplified length fragment polymorphisms (AFLPs) and 18 paired interspersed nuclear elements (PINEs) primer combinations. I was not able to detect a sex-linked marker with this method, possibly due to the size and complexity of the sex determining region, and the similarity of the sex chromosomes to one another.



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CHAPTER III





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# Sex-Linked Markers in Pink Salmon (*Oncorhynchus gorbuscha*)

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Several mechanisms controlling sex determination have evolved in plants and animals. Many marine invertebrates and plants are cosexual (Charlesworth 1991). In contrast, most terrestrial animal species are dioecious, where male and female gametes are produced by separate individuals. In such animals, an individual's sex may be determined by environmental cues, the presence or absence of egg fertilization (haplo-diploidy; e.g. ants, bees, and wasps), the presence of a sex determining gene or group of genes (polyfactoral), or by specialized sex chromosomes (Bull 1983, Rice 1996). Sex chromosomes are more common in animals than in plants and are believed to have evolved from autosomes (Rice 1996). Vertebrate sex chromosomes appear to have evolved independently in many lineages (Rice 1996, Saxena et al. 1996, Fridolfsson et al. 1998).

The process of Y-chromosome evolution begins when the progenitor Y-chromosome evolves a dominant, genic factor that determines gender. This factor may be a single, dominant gene or a collection of tightly linked genes.



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There is subsequent lack of recombination between the X-chromosome and the region containing the sex determining factor on the Y-chromosome (Charlesworth 1991, Rice 1996). Recombination is further suppressed due to the evolution of sexually antagonistic alleles that are tightly linked with the sex determining factor (Fisher 1931, Bull 1983, Rice 1987, Rice 1997).

Once suppressed recombination has occurred, the sex determining region is expected to accumulate mutations and chromosomal changes due to relaxed selection. Presumably, the X-chromosome contains functional copies of genes also found on the Y-chromosome that compensate for Y-linked gene degeneration. Additionally, the Y-chromosome will never be in the homozygous state in an individual for selection to eliminate the deleterious changes. Mutation rates are expected to be higher for Y-linked genes compared to X-linked genes due to the greater number of germ cell divisions required for spermatogenesis relative to oogenesis (Haldane 1947).

In addition to higher mutation rates, the Y-chromosome may also accumulate different types of repetitive elements. Transposable elements have been found on the Y-chromosome in *Drosophila simulans* and *D. melanogaster* (Junakovic et al. 1998), and a short interspersed nuclear element (SINE) has been reported in *Zfy* (zinc finger of the Y) in seven domestic cat lineages (Slattery and O'Brien 1998). In

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humans and other higher apes, an *Alu* element has been detected at the boundary between the pseudoautosomal and sex-chromosome specific region of the Y-chromosome (Ellis et al. 1990).

The differential segment on the Y-chromosome containing the sex determining region continues to grow due to the accumulation of mutations, repetitive elements, and sexually antagonistic alleles, and lack of recombination with the X-chromosome. Eventually, a chromosomal sex determining system is reached once the differential Y segment constitutes most of the chromosome except for the pseudoautosomal region that pairs with and undergoes recombination with the X-chromosome during meiosis (Burgoyne 1986, Ellis et al. 1990).

Sex determination in fish species is rather complex. Hermaphroditism is present in species from at least 14 families of teleost fishes (Moyle and Cech 1996). In most cases, these species are transitional or sequential hermaphrodites, most commonly where a female turns into a male (often when the dominant male of a harem dies). Environmental sex determination, where the temperature of the water determines the sex of the individuals is also present in many species. The first evidence for a genetic basis of sex determination came via the discovery of a sex-linked color factor in the medaka (*Oryzias* (formerly *Aplocheilus*) *latipes* ; Aida 1921). Later, studies with the

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guppy (*Poecilia reticulatus*), found that ornamental characteristics in male guppies were controlled by 18 genes, 17 of which were perfectly linked with the sex determining factor (Winge 1927).

The evolution of a chromosomal sex determining system has occurred independently and at different times in several fish species (Kirpichnikov 1981, Price 1984, Nanda et al. 1992, Rice 1996). Hermaphroditism appears to have been the ancestral state in fish, with a genic sex determining system evolving later (Kirpichnikov 1981). Previous work suggests that many of the teleost fishes, including salmonids, are in a primitive state of chromosomal sex determination.

Fish in the family Salmonidae descended from a single tetraploid ancestor 25-100 million years ago (Allendorf and Thorgaard 1984). Salmonids have been found to have a XX female, XY male sex determining system based on chromosomal studies (Thorgaard 1977, Thorgaard and Gall 1979) and breeding studies with sex-reversed fish (Okada et al. 1979, Johnstone et al. 1979). In triploid rainbow trout (*Oncorhynchus mykiss*), XXY fish are males, providing evidence for a dominant Y sex determining factor (Allendorf and Thorgaard 1984).

Unlike the mammalian system of full chromosomal sex determination with a small pseudo-autosomal region, salmonids show few genetic differences between the sex chromosomes. The viability of YY males in coho salmon (*O.*

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*kisutch*; Hunter et al. 1982), and the viability and fecundity of YY hermaphrodites in rainbow trout (Chevassus et al. 1988) support the existence of functional genes on the Y-chromosome, and slight genetic differentiation between the X and Y. Due to the lack of genetic differences observed, recombination between the X and Y chromosomes must still occur. Indeed, recombination between the sex chromosomes was observed in the cross used for a linkage map study in rainbow trout (Young et al. 1998).

Sex chromosomes in the family Salmonidae are largely isomorphic. While the karyotypes of many species in the Salmonidae family are currently known, most species show little difference between the X and Y chromosomes in both staining and morphology (Frolov 1993). The sex chromosomes in rainbow trout (Thorgaard 1983), lake trout (*Salvelinus namaycush*; Phillips and Ihssen 1985, Frolov 1993), and sockeye salmon (*O. nerka*; Thorgaard 1978, Frolov 1990) have shown some differentiation cytogenetically. However, the inability to distinguish the X and Y chromosomes in the majority of species indicates the sex chromosomes are in an early state of evolution.

It has been difficult to detect sex-linkage in salmonids, due to the presence of functional loci found on both the X and Y chromosomes. Males express both X and Y-linked copies of alleles making it difficult to distinguish autosomal loci from those present on the X or Y chromosomes.

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Whereas the term "sex linked" usually refers to loci found only on the X-chromosome, loci that are sex linked in salmonids refer to those present on either X or Y chromosome in an area outside the pseudoautosomal region.

Little is understood about the sex determining region (*SEX*) in salmonids, and relatively few sex-linked markers are known. Earlier studies in rainbow trout placed *SEX* near the centromere through gene-centromere mapping (Allendorf et al. 1986) and cytogenetic studies (Thorgaard 1977, Lloyd and Thorgaard 1988). A linkage map recently developed for rainbow trout mapped *SEX* to a more distal position on the short arm of a subtelocentric chromosome (Young et al. 1998). *SEX* is also thought to reside on the short arm of a subtelocentric chromosome in lake trout (Reed et al. 1995). Using inheritance data to calculate recombination rates, two sex-linked allozyme loci, *HEX-2* and *sSOD1* were detected in rainbow trout (Allendorf et al. 1994). In some chinook salmon (*O. tschawytscha*) populations, the allozyme *PEPB-1* is sex-linked based on inheritance data (Marshall, et al. in preparation). In addition, joint segregation analysis on sparctics, a hybrid between brook trout (*S. fontinalis*) and Arctic char (*S. alpinus*), detected tight linkage between *SEX* and three allozyme loci, *LDH-1*, *AAT-5*, and *GPI-3* in brook trout (May et al. 1989).

In addition to allozymes, other sex-linked markers have

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been detected in salmonids. A male-specific DNA probe (OtY1) has been developed for chinook salmon using subtractive DNA hybridization (Devlin et al. 1991). Further work has shown that the OtY1 probe is located within a tandemly repeated 8 kb unit comprising about 2.4 Mb located on the Y-chromosome (termed OtY8; Devlin et al. 1998). The repeat unit, or similar sequences, are present in all closely related species but are not sex-specific (Devlin et al. 1998).

In brown trout, (*Salmo trutta* L.) tight linkage between *SEX* and the minisatellite probe *Str*-A9, developed from a brown trout DNA phagemid library, was detected using joint segregation analysis (Prodohl et al. 1994). In rainbow trout, fluorescent *in situ* hybridization (FISH) was used to demonstrate an X-linked copy of 5S rDNA (Moran et al. 1996). Using FISH, females exhibited four hybridization signals, while males had three, indicating 5S rDNA is duplicated and located on both an autosomal metacentric chromosome pair and on the subtelocentric X-chromosome (Moran et al. 1996). Finally, a Y-linked growth hormone pseudogene has been detected in some *Oncorhynchus* species (Forbes et al. 1994, Kavsan et al. 1994, Du et al. 1993).

The majority of sex-linked loci observed in salmonid species thus far appear to be largely taxon-specific. The apparent lack of sex-linked orthologous loci suggest that either these loci are monomorphic in closely related taxa

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and sex-linkage cannot be observed in inheritance studies, or taxon-specific karyotype differences exist and *SEX* evolved in different genomic regions.

Sex-linked markers can provide important genetic information. Sex-linked markers are useful for identifying the gender of immature fish, or fish that have been hormonally treated. Sex-linked genetic markers can be used to investigate the genealogy and phylogeny of species, for gene mapping, and to detect geographic population structures. Hybridization between species can often be detected through Y-introgression of markers. Allele frequencies at sex-linked loci differ from autosomal loci, and in salmonids, where diploidization is taking place (Allendorf and Danzmann 1997a), tetrasomic versus disomic inheritance can be evaluated with sex-linked loci. Sex-linked markers can also help evaluate rates of recombination between the X and Y chromosomes in males, the X and X chromosomes in females, and can help determine how large the non-combining, sex determining region of the Y-chromosome is.

Many species of Pacific salmon are currently threatened or endangered (Nehlsen et al. 1991, National Research Council 1996, Allendorf et al. 1997b). While pink salmon are not endangered, populations have disappeared from coastal Washington, Oregon, and California (National Research Council 1996), and this species is susceptible to

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habitat degradation (e.g. the March 1989 *Exxon Valdez* oil spill). Pink salmon (*O. gorbuscha*) are currently the most abundant anadromous salmon in North America (National Resource Council 1996). They have an extensive range, spawning in Asian and North American streams bordering the Pacific and Arctic oceans, and are the most numerous salmon in the commercial salmon fisheries of this region, important to the fisheries of Japan, Russia, Canada, and the United States (U.S. Fish and Wildlife Service 1989).

Currently, no sex-linked markers have been described in pink salmon. Consequently, the main objective of this thesis research was to detect and describe sex-linked genetic markers in pink salmon. The work presented here is the results from a two-year study aimed at detecting sex-linked markers in pink salmon using a variety of molecular methods.

The goal of chapter 2 was to detect a pseudogene derived from growth hormone 2 in pink salmon. This pseudogene (*GH-2p*) has previously been found to be perfectly linked to the sex determining region on the Y-chromosome in chinook salmon (Du et al. 1993), coho salmon (Forbes et al. 1994), and chum salmon (*O. keta*; Kavsan et al. 1994). In addition to testing for the presence of the growth hormone pseudogene (*GH-2p*) in pink salmon males, the third intron (intron C) of *GH-2* was also investigated in pink salmon to

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be sure the pseudogene was not being confused with amplification of *GH-2*.

The goal of chapter 3 was to detect an additional sex-linked marker in pink salmon to complement an ongoing genome mapping project of this species (Allendorf et al. 1997c, Allendorf et al. 1998, Spruell et al. 1999). Whereas the growth hormone derived pseudogene is perfectly linked to the sex determining region on the Y-chromosome, the goal of the research presented in chapter 3 was to detect a marker closely linked to *SEX* but located in the pseudoautosomal region. Such a marker will also be present on some X-chromosomes, and may make it possible to identify the X-chromosome linkage group. In order to target a marker linked to *SEX*, bulked segregant analysis was used to screen multiple loci. This method allows comparison of two groups of DNA that segregate for a trait of interest, in this case sex. Combining DNA from male and female pink salmon from the same family, creates two pools of DNA that theoretically differ only in their sex chromosome composition. Two classes of multi-locus genetic markers; AFLPs (amplified fragment length polymorphic DNAs) and PINEs (paired interspersed nuclear elements) were screened on the pooled DNA in order to detect a marker closely linked to *SEX*.

Finding a sex-linked marker such as an AFLP or a PINE may make it possible to identify the sex chromosome linkage group on the map. Yet, even if the marker is not able to be

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"linked up" to the rest of the markers on the current linkage map, an additional sex-linked marker detected in pink salmon would be valuable and useful for genetic studies in this species. In addition, such a sex-linked marker could be potentially valuable for application in other Pacific salmon species, or alternatively provide additional evidence for the lack of sex-linked orthologous loci present within salmonids.

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# Detection of a Y-Linked Pseudogene in Pink Salmon (*Oncorhynchus gorbuscha*)

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**ABSTRACT**

I amplified and sequenced a portion of an intron from a Y-linked growth hormone pseudogene in pink salmon (*Oncorhynchus gorbuscha*). This pseudogene provides an unambiguous, PCR-based test for sex in pink salmon, and is the first Y-linked marker described in this species. A duplication event of the functional growth hormone 2 (*GH-2*) gene gave rise to the growth hormone 2 pseudogene (*GH-2p*). The third intron (intron C) of *GH-2* was also amplified and sequenced in pink salmon to be certain that amplification of *GH-2p* was not confounded by *GH-2*. A length polymorphism was detected in intron C of *GH-2* and is due to an 81 bp insertion similar to the 3' end of the *Sma*I SINE (short interspersed nuclear element). Phylogenetic analysis of intron C sequence data from *GH-2* and *GH-2p* in pink salmon and other salmonids suggests that the gene duplication event giving rise to *GH-2p* occurred after the split of *Salmo* and *Oncorhynchus* but before rainbow trout and cutthroat trout



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diverged from the other *Oncorhynchus* species.

## INTRODUCTION

Growth hormone (somatotrophin) plays an important role in many physiological and biochemical functions in salmon. Fish growth hormone is a 20-22 Kilodalton single chain polypeptide hormone, expressed in the somatotrophs of the anterior pituitary (Du et al. 1993, Yang et al. 1997). Growth hormone stimulates protein synthesis and promotes lipid and glycogen breakdown (important during migration to spawning sites when the fish do not eat). In addition, growth hormone is the primary regulator of somatic growth and is involved in sexual maturation (Bjornsson 1997). Growth hormone is also important in the anadromous life cycle of salmon, improving hypoosmoregulation in fresh water and increasing tolerance to sea water (Bjornsson 1997).

Salmonids descended from progenitors in which a single, auto-tetraploidization event occurred between 25-100 million years ago (Allendorf and Thorgaard 1984). As a result of this tetraploid event, salmonids have two functional, autosomal, growth hormone genes, *GH-1* and *GH-2* (Devlin 1993, Forbes et al. 1994, Yang et al. 1997). Both these genes have been sequenced and characterized using cDNA GH clones in a variety of species including Atlantic salmon (*Salmo salar*; Johansen et al. 1989, Male et al. 1992), coho salmon

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(*Oncorhynchus kisutch*; Nicoll et al. 1987), chum salmon (*O. keta*; Kawauchi et al. 1986), chinook salmon (*O. tschawytscha*; Du et al. 1993), sockeye salmon (*O. nerka*; Devlin, R. H. 1993), and rainbow trout (*O. mykiss*; Yang et al. 1997).

In comparison with other vertebrates such as mammals and birds, and with other fish species such as carp, flounder, and bass, salmonids are the only group known to have two, separate, functional genes coding for growth hormone (Yang et al. 1997). Both genes are structurally similar to the growth hormone gene of other species, but lack an intron (intron E) that splits the terminal exon of other species, resulting in six exons and five introns in salmonids (Yang et al. 1997). The functions of both growth hormone genes is not precisely known. Two-year old, female rainbow trout have higher levels of *GH-1* mRNA than of *GH-2*, while this difference in not seen in males. Rainbow trout fry also appear to express higher levels of *GH-1* than *GH-2*, although a sex-specific difference can not be determined at this stage (Yang et al. 1997).

In addition to the two, autosomal growth hormone genes, a later gene duplication of *GH-2* gave rise to a growth hormone pseudogene (*GH-2p*; Du et al. 1993). Pseudogenes are DNA segments with a high degree of homology with functional genes, but contain nucleotide changes that prevent their

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translation (Li et al. 1981, Li 1997). Pseudogenes have been detected in a wide-range of taxa, providing evidence for the widespread occurrence of both gene duplication, and loss of gene function due to relaxed selective constraints (Li et al. 1981). Pseudogenes can evolve by either a gene duplication event, or can arise by reverse transcription of RNA (termed processed pseudogenes).

The growth hormone pseudogene has been detected in three salmonid species: chinook salmon (Du et al. 1993), coho salmon (Forbes et al. 1994), and chum salmon (Kavsan et al. 1994). The pseudogene has been detected only in the males of these species, and appears to be perfectly linked with the sex determining region on the Y-chromosome. This pseudogene contains introns and therefore evolved through gene duplication rather than reverse transcription. The complete nucleotide sequence of the pseudogene in chinook salmon has been determined (Du et al. 1993). The pseudogene spans 5.5 kb and although it has a proper TATA box, it contains a premature termination codon, a 150-bp deletion in the last half of exon 5, and a wrong splicing signal at the intron A/exon 2 junction that precludes correct splicing and translation (Du et al. 1993).

In chum salmon, the pseudogene has been sequenced up through the third intron (intron C; Kavsan et al. 1994). The pseudogene shows close homology with the functional *GH-2* from rainbow trout for the first intron, second exon, and

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second intron (although nucleotide mutations prevent correct splicing of the first intron, and a premature stop codon is found in the second exon). The third exon, and beginning of the third intron (intron C) are deleted in this species, and are partially replaced by a 83 bp A-T rich region (Kavsan et al. 1994).

The purpose of this paper was to test if *GH-2p* is both present and Y-linked in pink salmon and could be useful as a sex-linked marker in this species. Intron C of *GH-2* was also investigated in pink salmon to be sure that detection of the pseudogene was not confounded by amplifying the functional counterpart. The results of amplifying and sequencing a portion of intron C from *GH-2p* and intron C from *GH-2* are presented. Phylogenetic analysis was performed using sequence data from intron C of *GH-2* and *GH-2p* to determine when the duplication event leading to the pseudogene occurred.

## MATERIALS AND METHODS

### Samples and Haploid Gynogenesis

In August of 1995, gametes and tissues of 31 adult (based on sexual maturity) pink salmon were collected from the Armin F. Koernig hatchery, Prince William Sound, Alaska. Seven families of gynogenetic haploid embryos were produced by sperm inactivation as described by Thorgaard et al.

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(1983). Sperm from four males were pooled and irradiated with UV light and mixed with the eggs from individual females. Females were numbered and their progeny were designated by year class and the number assigned to the female (e.g. family 95-105). Embryos were incubated until just prior to hatching when they were collected and preserved in ethanol.

Gametes and tissues were also collected from 37 adult pink salmon from the 1996 year class in August 1996, and were used to make normal diploid as well as gynogenetic haploid families. Chum salmon tissue samples taken from throughout their range (10 individuals from each of nine populations) were kindly provided by Penny Crane with the Alaska Department of Fish and Game.

DNA was isolated from muscle or liver tissue in the adults, and from the embryos after separation from the yolk sac in the progeny using the Purgene(™) DNA isolation kit (Genta Systems Inc.) The concentration of DNA was determined using a scanning spectrofluorometer.

*GH-2*

Primers designed from conserved positions in exons 3 and 4 in coho salmon, chum salmon, and rainbow trout, (Forbes et al. 1994) were used to amplify intron C of *GH-2* in pink salmon. Polymerase chain reaction (PCR) was performed in mixtures that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5.0 mM $MgCl_2$, all four dNTPs (each at 0.2

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mM), each primer at 0.3 *p*M, and 0.08 unit of *Taq* DNA polymerase (Perkin-Elmer/Cetus), and 40 ng of template DNA in a total volume of 15 μl. The PCR profile was 30 cycles of 92°C for 1 min, 63°C for 1 min, and 72°C for 2 min. PCR products were electrophoresed on 2.0% agarose gels in TAE buffer (Ausubel et al. 1989), containing ethidium bromide at 0.5 μg/ml.

***GH-2* Pseudogene**

The forward primer used to amplify a portion of intron C of the Y-linked pseudogene, 5'-TTTCTCTACGTCTACATTCT-3', was kindly provided by R. H. Devlin. This primer was designed from *GH-2* sequence from chinook salmon and lies 324 bp within intron C (Fig. 1). The reverse primer was the same as that used to amplify *GH-2* (Fig. 1). PCR and gel electrophoresis conditions were the same as for *GH-2*, except 5.8 mM $MgCl_2$ and an annealing temperature of 51°C were used.

**Sequencing and Analysis**

Four gynogenetic haploid pink salmon derived from female 95-115 were used to sequence intron C of *GH-2*. Two male pink salmon from the 1995 adult sample were used to sequence intron C of *GH-2p*. For sequencing of the pseudogene, PCR products were first run out on a Metaphor agarose gel to separate the pseudogene from the *GH-2* products, and then were subjected to a second round of PCR. PCR products for sequencing were purified from agarose gels

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using the GENECLEAN kit (BIO 101 Inc.) and sequenced by direct automated sequencing (Applied Biosystems Inc.). These sequences, and other intron C sequences from *GH-2* and *GH-2p* from several other salmonids (Table 1) were aligned using Sequencher version 3.1 (Genecodes Corp., Ann Arbor MI).

Phylogeny reconstruction was performed using the methods of parsimony, maximum likelihood, and neighbor joining available within PAUP* version 4.0.0d64 (Swofford 1999). Parsimony analysis was performed using Fitch parsimony (1971) with unordered characters, equal weighting and using the branch-and-bound algorithm. Bootstrap values represent the percentage of 1000 replicates that supported a particular branch. Branch lengths were analyzed using MacClade version 3 (Maddison and Maddison 1992).

Phylogenetic analysis was performed using nucleotide sequence data from intron C of *GH-2* in nine species, and intron C of *GH-2p* in four species (Table 1). Atlantic salmon was chosen as the outgroup because the ancient split of *Salmo* and *Oncorhynchus* has been established (Stearley and Smith 1993, McKay et al. 1996, Murata et al. 1996), and this allowed resolution of the *Oncorhynchus* salmonid sequences. Brown trout (*Salmo trutta*) was included to help provide further resolution (excluding this species resulted in 15 most parsimonious trees). Because of the potential problems

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associated with taxa not having complete sequence information, phylogenetic analysis was also performed after removing the pink salmon and chum salmon *GH-2p* sequences.

## RESULTS

### *GH-2*

Intron C of *GH-2* was amplified in 31 pink salmon collected in 1995, and 37 collected in 1996. The *GH-2* intron C primers amplified two products in pink salmon of 540 bp and 621 bp in length. The product lengths correspond to intron C, 17 bp of exon 3, 29 bp of exon 4 (since the priming sites lie within exon 3 and exon 4), and the primers (24 bp each). Sequencing both alleles revealed the intron lengths specifically to be 446 bp and 527 bp in length (Fig. 1). These alleles therefore have been named *GH-2*C446* and *GH-2*C527* to reflect intron length (Spruell et al. 1999; Genbank No. AF075571, AF075572).

For the 1995 year class, seven of 31 individuals were heterozygotes, and the remaining 24 individuals were homozygotes for the *GH-2*C446* allele (Table 2). In the 1996 year class sample, eight of 37 individuals were heterozygotes, and the remaining 29 individuals were homozygotes for the *GH-2*C446* allele (Table 2). There were no individuals homozygous for the *GH-2*C527* allele present in either year class sample. Both odd and even-year class

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samples were in Hardy Weinberg proportions at this locus, (Table 2). A sex-specific difference in genotypes and allele frequencies at *GH-2*C* was not observed.

The allelic nature of these products was tested by looking at segregation patterns in gynogenetic haploids. Two females heterozygous for *GH-2*C* and their gynogenetic haploid progeny were tested for inheritance at *GH-2*C*. Thirty-six haploid progeny from female 95-115 and thirty-six haploid progeny did not significantly differ (at p = .05) from expected 1:1 Mendelian segregation for these alleles based on a two-tailed test of binomial probabilities (Table 3).

Aligning sequences using the Sequencher program, showed that alleles *GH-2*C446* and *GH-2*C527* differed by an 81 bp insert in *GH-2*C527*. This insert is nearly identical to the 3' end of the consensus sequence of the *Sma*I SINE (short interspersed nuclear element; Fig. 2) found in the genomes of pink salmon and chum salmon (Kido et al. 1991).

Because pink salmon and chum salmon are the only salmonids to have the *Sma*I element present in their genomes, 90 chum salmon were also tested for the presence of the *Sma*I insert. Using the primers designed to amplify intron C of *GH-2*, a single 601 bp product was amplified in all samples, which is what is expected according to published *GH-2* chum salmon sequence data (Fig. 4). There was no evidence for a

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*GH-2* allele containing the *Sma*I insert in any of the chum salmon tested.

**_GH-2_ Pseudogene**

Known male and female pink salmon (based on sexual maturity) were analyzed using the pseudogene primers. Twelve males and 19 females from the 1995 year class, and 15 males and 22 females from the 1996 year class were tested. Using the pseudogene primers, all known males from both year classes preferentially amplified a 166 bp product from *GH-2p* (Fig. 3). Males also weakly amplify a portion of the *GH-2*C446* and *GH-2*C527* alleles (depending on genotype), and a 213 bp product from intron C of *GH-1* (Fig. 3). Using the pseudogene primers, all known females from both year classes amplify a portion of the *GH-2*C446* and *GH-2*C527* alleles, and a 213 bp product from intron C of *GH-1* (Fig. 3). Gynogenetic haploids generated from a female heterozygous at *GH-2*, segregate for the *GH-2* alleles as well as amplify a portion of intron C of *GH-1* (Fig. 3) when amplified using these primers.

All 27 known males tested using the pseudogene primers amplified the 166 bp product of *GH-2p*. All 41 known females tested lacked the pseudogene. The 122 bp of intron C and the beginning of exon 4 sequenced from *GH-2p* show close homology with the *GH-2*C446* and *GH-2*C527* alleles (Fig. 1).

**Phylogenetic Analysis**

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Phylogenetic analysis was performed using intron C data from *GH-2* and *GH-2p* to determine when the pseudogene arose. All three tree-building methods availiable within PAUP* version 4.0.0d64 (parsimony, likelihood, and neighbor-joining) produced trees with similar topologies. A single most parsimonious tree was generated using the method of parsimony and is shown in Fig. 5. Analysis performed after removing the pink salmon and chum salmon pseudogene sequences (in case incomplete sequence data was a confounding factor) did not change the tree topology or the relationships of the taxa from the tree in Fig. 5, except that the pseudogene sequences from coho salmon and chinook salmon clustered together. The relationships of coho salmon, chinook salmon, pink salmon, chum salmon, sockeye salmon, rainbow trout and cutthroat trout generated from intron C sequence data of *GH-2*, agree with published trees for these species based on the combined evidence from intron D of *GH-2*, as well mtDNA NADH dehydrogenase subunit 3 (Fig. 6).

Phylogenetic analysis using intron C of *GH-2* and *GH-2p* results in the pseudogene sequences forming a single clade, branching off before any of the *Oncorhynchus GH-2* sequences. This indicates that the gene duplication event of *GH-2* occurred after the split of *Salmo* and *Oncorhynchus*, but before the divergence of rainbow and cutthroat from the

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Pacific salmon. While the bootstrap value lends weak support (62) to this branch, the overall tree topology is consistent and suggestive. In comparing the branch lengths for species with sequence data for both *GH-2* and *GH-2p*, branch lengths are longer for the pseudogenes in chinook salmon, pink salmon, and chum salmon (Fig. 5). This is consistent with pseudogenes having a higher rate of nucleotide substitution in comparison with different parts of other genes (Li 1997).

## DISCUSSION

### *GH-2*

Growth hormone 1 and 2 have previously been characterized using a number of species including rainbow trout (Yang et al. 1997, Agellon et al. 1988), Atlantic salmon (Johansen et al. 1989, Male et al. 1992), sockeye salmon (Devlin 1993), and chinook salmon (Du et al. 1993). Using PCR, a single amplicon is produced for intron C at *GH-2* in coho, and chinook salmon. While no allelic differences in intron C of *GH-2* has been detected by length in rainbow trout, SSCP (Single strand conformational polymorphism) analysis has detected up to five alleles in a single population (Bagley and Gall 1998).

Pink salmon have two distinct alleles (based on length) at intron C of *GH-2*. These alleles, *GH-2*C446* and *GH-2*C527*

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are found in both odd and even-year pink salmon runs with identical frequencies (Table 2). Both odd and even-year classes showed no significant deviations from expected Hardy-Weinberg proportions (Table 2). Sequencing revealed that the size difference of the alleles is due to an 81 bp insert that is nearly identical to the 3' end of the *Sma*I element.

SINEs are repetitive sequences found throughout the eukaryotic genome (Okada 1991). SINEs are less than 500 bp in length, and are characterized by having a tRNA-related region, and internal RNA polymerase III promoter, and an AT rich region (Okada 1991, Spruell and Thorgaard 1996, Li 1997). SINEs are amplified by reverse transcription, and appear to be inserted irreversibly into various sites throughout the genome (Takasaki et al. 1997).

The two-year life cycle of pink salmon is so strict that odd and even year fish are genetically isolated (Hart 1973, Donnelly 1983), such that pink salmon differ more between odd and even year runs than between fish within odds and evens throughout their range (Phillips and Kapuscinski 1988). The presence and frequency of the *GH-2*C446* and *GH-2*C527* alleles in both year classes suggest the *Sma*I insert occurred before pink salmon year classes evolved.

The *Sma*I element has been found to be restricted to the genomes of pink salmon and chum salmon (Takasaki et al. 1997, Greene and Seeb 1997). It might be expected that the

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insertion of the 3' end of *Sma*I into *GH-2* occurred in the common ancestor of pink and chum salmon (around 6 million years based on fossil evidence; McKay et al. 1996). However, chum salmon tested from throughout their geographic range provided no evidence for the presence of the *Sma*I insert in *GH-2*. Thus the insertion seems to have occurred after pink salmon diverged from chum salmon, but before the year classes evolved. A previous study of the interspecific and intraspecific variation of *Sma*I found a lack of shared insertion sites between the genomes of pink salmon and chum salmon (Takasaki et al. 1997). The authors offer several possible explanations for this result such as horizontal gene transfer, introgression, and temporal differences in amplification within lineages. While horizontal gene transfer and introgression cannot be ruled out, it also seems plausible that the *Sma*I family evolved in the common ancestor of pink salmon and chum salmon, and underwent differential amplification and distribution in the genomes after these two species diverged.

It is interesting that only a portion (the 3' end) of the *Sma*I element is found inserted within *GH-2*C527*. Previous studies have also observed that some regions of SINEs may be distributed independently of the remainder of the element. Spruell and Thorgaard 1996, detected different DNA fingerprint patterns in 14 species of salmonid fishes

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when they used probes matching different regions of the *Hpa*I and *Fok*I SINEs. A genetic linkage map of rainbow trout found that the same locus was never detected using probes of alternative ends of the *Hpa*I SINE were used (Young et al. 1998). It appears that SINEs are more complex than originally thought. Caution should be taken when using such elements for phylogenetic analysis since their patterns of distribution in the genome and mode of insertion is not fully clear.

***GH-2* Pseudogene**

The growth hormone pseudogene is Y-linked in pink salmon as it is in chinook salmon, coho salmon, and chum salmon. All known pink salmon males tested from both odd and even-year classes strongly amplified a pseudogene-specific product when primers designed to amplify the pseudogene were used. In addition, males weakly amplify the *GH-2*C* alleles as well as a portion of intron C from *GH-1*, which is expected based on the close homology of *GH-2*, *GH-2p*, and *GH-1* in the priming sites. Female pink salmon amplify one or both *GH-2*C* alleles depending on their genotype, as well as a portion of intron C from *GH-1*.

This Y-linked growth hormone pseudogene is the first known sex-linked marker described in pink salmon. The ability to detect the pseudogene using PCR makes the genetic test for sex in immature pink salmon, and of tissue or DNA

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samples of unknown origin both cost effective and time efficient. Moreover, the test is extremely reliable and dependable. Since PCR products are amplified in females as well as males, the test for the presence of the pseudogene will not be confounded by a failed PCR reaction.

The nucleotide sequence for the 93 bp from *GH-2p* shows close homology with that of *GH-2*C446* and *GH-2*C527* (Fig. 1). Previous attempts to detect the pseudogene in pink salmon by amplifying the entire intron and using restriction enzymes to differentiate *GH-2*C* from *GH-2p* (as was done in coho salmon; Forbes et al. 1994) were unsuccessful (F. W. Allendorf personal communication; R. H. Devlin personal communication). This indicates that in *GH-2p*, the upstream region of intron C (from where the sequence begins (Fig. 4)), and possibly exon 3 may be deleted in pink salmon as it is in chum salmon.

It is clear that the growth hormone pseudogene is closely linked to the sex determining region on the Y-chromosome, although it is unclear how physically close the pseudogene may be. A chromosomal walk initiated from *GH-2p* in chinook salmon produced cosmids containing mostly repetitive DNA that does not show sex-specificity with other salmonid species (Devlin et al. in preparation). Allele frequencies observed for male and female pink salmon at *GH-2*C* are similar despite small sample sizes suggesting *GH-2*

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is not closely linked with the sex determining region in pink salmon. Previous segregation studies in coho salmon and chinook salmon (Devlin et al. in preparation), demonstrated that *GH-2* is not X-linked in these species, suggesting that the pseudogene may have arisen from a chromosomal rearrangement rather than divergene of one copy of a sex-linked locus.

**Phylogenetic Reconstruction**

All methods of reconstructing phylogeny based on sequence data from intron C of *GH-2* and *GH-2p* produced the same overall tree topology. The *GH-2* duplication event giving rise to *GH-2p* appears to have occurred after the spilt of Atlantic and Pacific salmon (at least 19.9 million years ago; McKay et al. 1996), but before rainbow and cutthroat diverged from the other Pacific salmon. This agrees with the findings of Du et al. 1993 as to the timing of the emergence of the pseudogene. The four pseudogenes form their own clade, suggesting that *GH-2p* likely diverged before the species (i.e. chinook salmon, coho salmon, pink salmon, and chum salmon) diverged from one another.

However, the different rate of nucleotide substitution, and the subsequent long branches in the pseudogene clade may be a potential source of error in reconstructing phylogenies due to long-branch attraction. In long-branch attraction, long branches of a tree may be randomly attracted to each

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other and confound a phylogenetic signal (Felsenstein 1978). This could be the reason the pseudogene clade clusters closely with the outgroup (Fig. 5), which is also a long branch. However, if there is a problem with long-branch attraction in the data set, the pseudogene clade should also be randomly attracted to the long branch leading to the coho salmon and chinook salmon *GH-2* branch, which is not observed. This suggests a true phylogenetic signal is present and the pseudogene arose after *Oncorhynchus* and *Salmo* diverged, but before the seven Pacific salmonids tested diverged from one another.

Attempts to amplify the pseudogene in sockeye salmon, rainbow trout, and cutthroat trout have been unsuccessful to date (Du et al. 1993, Forbes et al. 1994, R.H. Devlin personal communication). It is possible that the pseudogene is still present in these species, but is unable to be amplified using PCR techniques due to mutations in the priming sites. Indeed, primers used to amplify the pseudogene in coho salmon and chinook salmon failed to amplify the pseudogene in chum salmon due to a deletion in the priming site in exon 3 (Kavsan et al. 1994). Alternatively, the pseudogene may not be detected in these species because the pseudogene has been excised. The apparent absence of *GH-2p* in sockeye salmon may be due to a loss of this region during a Y-autosomal robertsonian translocation (Thorgaard 1978, Frolov 1993).

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Pseudogenes are now thought to be extremely common in most taxa (Li 1997). Gene duplication allows a gene to be free of selection, and since a normal gene is still functioning, the loss or change in function of one of the pairs is common. Since pseudogenes are free from selective constraints, all nucleotide substitutions are neutral and will become fixed or lost in a population by genetic drift. Pseudogenes have been shown to evolve more rapidly (i.e. accumulate more mutations) than other regions of the genome (Li 1997). The longer branch lengths (corresponding to the amount of nucleotide substitution) observed for *GH-2p* in chinook salmon, pink salmon, and chum salmon compared with *GH-2*C* in these species (Fig. 5) is consistent with the expectation of higher rates of evolution in pseudogenes.

Results from *GH-2*C* in this study show pink salmon and chum salmon as being sister species, although bootstrap support for this branch is less than 50. The relationships among sockeye salmon, pink salmon, and chum salmon has been debated (see McKay et al. 1996 for review). In the past, sockeye salmon and pink salmon have been placed as sister species based on morphology, and the similarities observed in sequence data between pink salmon and chum salmon were thought to be due to hybridization between these species (Stearley and Smith 1993). Recent analysis of mtDNA (Domanico and Phillips 1995), and analysis of mtDNA combined with nuclear markers (McKay et al. 1996, Domanico et al.

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1997, Kitano et al. 1997) provide strong support for a sister relationship between chum and pink salmon (Fig. 6).

**Summary**

The growth hormone pseudogene is Y-linked in pink salmon. This pseudogene appears to be perfectly linked to the sex determining region in pink salmon (there is no recombination with the X-chromosome) and is therefore an important Y-linked marker in this species. Pink salmon have a length polymorphism in *GH-2*C* that is present in males and females of both odd and even-year classes. The length difference is caused by an insertion of a portion of the *Sma*I SINE into the longer allele and appears to be specific to pink salmon. The insertion appears to have happened after pink salmon and chum salmon diverged, but before the odd and even year classes of pink salmon evolved. The duplication of *GH-2* that led to *GH-2p* appears to have occurred in the genus *Oncorhynchus* after the split from *Salmo*, but before the rainbow trout and cutthroat trouts branched off from the other Pacific salmon.

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**Table 1.** Sequences used in phylogenetic analysis of intron C from growth hormone 2 (*GH-2*), and the growth hormone pseudogene (*GH-2p*).

| Species | Common name | Locus | Reference | Genbank No. |
|---|---|---|---|---|
| *Salmo salar* | Atlantic salmon | *GH-2* | Johansen et al. 1989 | M21573 |
| *Salmo trutta* | Brown trout | *GH-2* | | AF005912 |
| *O. mykiss* | Rainbow trout | *GH-2* | Agellon et al. 1988 | J03797 |
| *O. clarki* | Cutthroat trout | *GH-2* | | AF005913 |
| *O. tshawytscha* | Chinook salmon | *GH-2* | Du et al. 1993 | AF005914 |
| *O. kisutch* | Coho salmon | *GH-2* | Forbes et al. 1994 | U04930 |
| *O. keta* | Chum salmon | *GH-2* | | D16491 |
| *O. gorbuscha* | Pink salmon | *GH-2** | This study | AF075571 |
| *O. nerka* | Sockeye salmon | *GH-2* | Devlin 1993 | U14535 |
| *O. kisutch* | Coho salmon | *GH-2p* | Forbes et al. 1994 | U04931 |
| *O. gorbuscha* | Pink salmon | *GH-2p* | This study | |
| *O. tshawytscha* | Chinook salmon | *GH-2p* | Courtesy R.H. Devlin | |
| *O. keta* | Chum salmon | *GH-2p* | Kavsan, et al. 1994 | X74157 |

*Sequence data from allele *GH-2*C446* was used for phylogentic analysis

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**Table 2.** Observed and expected (parentheses) genotypes and allele frequencies of adult pink salmon from odd and even-year samples at *GH-2*C*. The *GH-2*C446* and *GH-2*C527* alleles have been abbreviated with the length (bp) of the alleles.

| | | Genotypes | | | Allele Frequencies | |
|---|---|---|---|---|---|---|
| Year Class | n | *446/446* | *446/527* | *527/527* | f(*446*) | f(*527*) |
| 1995 | 31 | 24 | 7 | 0 | .89 | .11 |
| | | (24.4) | (6.2) | (0.4) | | |
| 1996 | 37 | 29 | 8 | 0 | .89 | .11 |
| | | (29.4) | (7.1) | (0.4) | | |

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**Table 3.** Inheritance of *GH-2*C* in gynogenetic haploid progeny. The *GH-2*C446* and *GH-2*C527* alleles have been abbreviated with the length (bp) of the alleles.

| | | Progeny Genotype | | |
|---|---|---|---|---|
| Female | Genotype | *446* | *527* | p-val |
| 95-105 | *446/527* | 12 | 24 | 0.07 |
| 95-115 | *446/527* | 20 | 16 | 0.62 |

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**Fig. 1.** Aligned nucleotide sequence of intron C and a portion of flanking exons from *GH-2* and *GH-2p* from pink salmon. Unknown sequence is represented by ?; dashes represent gaps introduced to produce optimal sequence alignment. Stars indicate the priming sites for the pseudogene, the walls indicate the transition between exon and intron.

```
GH-2*C446  GAGATCAGAAGAGTTCA▌GTAAGTTACCTGGCTGAGACAATCCTCCATGATGCACAATTC  59
GH-2*C527  .................▌.........................................  59
GH-2p      ???????????????????▌?????????????????????????????????????

GH-2*C446  CAACATGAATAATAGGGCATCTCAATTTGAACAATCGATACAACTTAGTCATTAGTTATT 119
GH-2*C527  ............................................................ 119
GH-2p      ????????????????????????????????????????????????????????

GH-2*C446  GGGCAAGCAGATCCCCGATTGTCTAAACTCCATGGGTAAATATATACTGTAGATAAGAAG 179
GH-2*C527  ............................G............................... 179
GH-2p      ????????????????????????????????????????????????????????

GH-2*C446  AACCAGCATCATGCATGGTGGAAATTAAATCTAGCCATGACAGGGAGTTTTAAATTGTAC 239
GH-2*C527  ............................................................ 239
GH-2p      ????????????????????????????????????????????????????????

GH-2*C446  ACTTAAAAATCGGCAGGAAAATGTTGCTATACATCAGTGCCTTCAAAAACAACCACATTT 299
GH-2*C527  ............................................................ 299
GH-2p      ????????????????????????????????????????????????????????

GH-2*C446  CATAGTCATTGTAAGTAAAACCCATCACTCTCTAATTGGCGGTTTCTCTACGTCTACATT 359
GH-2*C527  ............................................................ 359
GH-2p      ????????????????????????????????????????**********************

GH-2*C446  CTGCAGCAATGTATCATG------------------------------------------- 377
GH-2*C527  .A..............TAAATAATATAATAATATAATAATATATGCCATTTAGCAGAC 419
GH-2p      **C....C.G........------------------------------------------  16

GH-2*C446  ----------------------------------------TAAATGATATGGCATCTCAAG 398
GH-2*C527  GCTTTTATCCAAAGCGACTTACAGTCATGTGTGCATACA..................... 479
GH-2p      ----------------------------------------..........T.........  37

GH-2*C446  CTGTACAATTACAACTCAACTTCATTTTCTAATAATCTGTGGTTTCTCTACATCTACACA 458
GH-2*C527  ............................................................ 539
GH-2p      .........-....TG.....................................-------.  88

GH-2*C446  CACAG▌GTCCTGAAGCTGCTCCATATCTCTTTCCG                          492
GH-2*C527  .....▌.........................                           573
GH-2p      .....▌............GT.G..........A************************  122
```

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**Fig. 2.** Aligned sequences of intron C for *GH-2* from sockeye (Devlin 1993) and pink salmon. The 81 bp insert found in *GH-2*C527* and the corresponding portion of the *Sma*I element are indicated by the dark bars in the sequence. The complete *Sma*I element is shown below the sequences; the region that corresponds to the *GH-2*C527* insert and its orientation is denoted by the arrow above the element. The solid shaded area corresponds to the tRNA-related region, the hatched region corresponds to the tRNA-unrelated region, and the open region is the AT rich region (Okada, 1991).

```
Sockeye    GTAAGTTACCGGGCTGAGACAATCCTCCATGATGCACAATTCCAACATGAATAATAGGGC  60
GH2*C446   ..........T.....................................................  60
GH2*C527   ..........T.....................................................  60

Sockeye    ATCTCAAGTTGAACAATCGATACAACTTAGTCATTAGTTATTGGGCAAGCAGATCCCCGA 120
GH2*C446   .......T..................................................... 120
GH2*C527   .......T..................................................... 120

Sockeye    TTGTCTAAACTCCATGGGTAAATATATACTGTAGATAAGAAGAACCAGCATCATGCATGG 180
GH2*C446   ..................................................................... 180
GH2*C527   ............G..................................................... 180

Sockeye    TAGAAATTAAATCTAGCCATGACAGGGAGTTTTAAATTGTACACTTAAAA-TCGGCAGGA 239
GH2*C446   .G.....................................................A..... 240
GH2*C527   .G.....................................................A..... 240

Sockeye    AAATGTTGCTATACCTCAGTGCCTTCAAAAACAACCACATGTCATAGTCCTTGTAAGTAA 299
GH2*C446   ............A.....................T........A.......... 300
GH2*C527   ............A.....................T........A.......... 300

Sockeye    AACCCATCACTCTCTAATCGGCGGTTTCTCTACGTCTACATTCTCCAGCAATGTGTCATG 359
GH2*C446   ..............T.....................G.........A..... 360
GH2*C527   ..............T.....................AG........A..... 360

Sockeye    TAAA|                                                        363
GH2*C446   ....|                                                        364
GH2*C527   ....|TAATA-TAATAATA-TAATAATATATGCCATTTAGCAGACGCTTTTATCCAAAGC 417
Sma I          |TAATAATAATAATAATA-TAATATATGCCATTTAGCAGACGCTTTTATCCAAAGC 105

Sockeye                                 |TGATATGGCATCTCAAGCTGTACAATTACAA 394
GH2*C446                                |............................. 395
GH2*C527   GACTTACAGTCATGTGTGCATACATAAA |............................. 476
Sma I      GACTTACAGTCATGTGTGCATACATTCT |

Sockeye    CTCAACTTCATTTTCTAATAATCTGTGGTTTCTCTACATCTACACACACAG          445
GH2*C446   .............................................               446
GH2*C527   .............................................               527
```


SmaI

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**Fig. 3.** Agarose gel showing PCR products from known males (lanes 1-3), known females (lanes 4 and 5), and gynogenetic haploids of female 5 (lanes 6 and 7). Known genotypes are based on prior amplification of *GH-2*C*. Male 1 is homozygous at *GH-2*, and amplifies the *GH-2*C446* allele, the Y-linked *GH-2p, and GH-1*. Males 2 and 3 are heterozygous at *GH-2*, and amplify both *GH-2* alleles, *GH-2p* and *GH-1*. Female 4 is hmozygous for *GH-2*C446*, and amplifies this allele and *GH-1*. Female 5 is heterozygous at *GH-2*, and amplifies both alleles as well as *GH-1*. Her haploids (lanes 6 and 7) segregate for the *GH-2* and amplify *GH-1*.

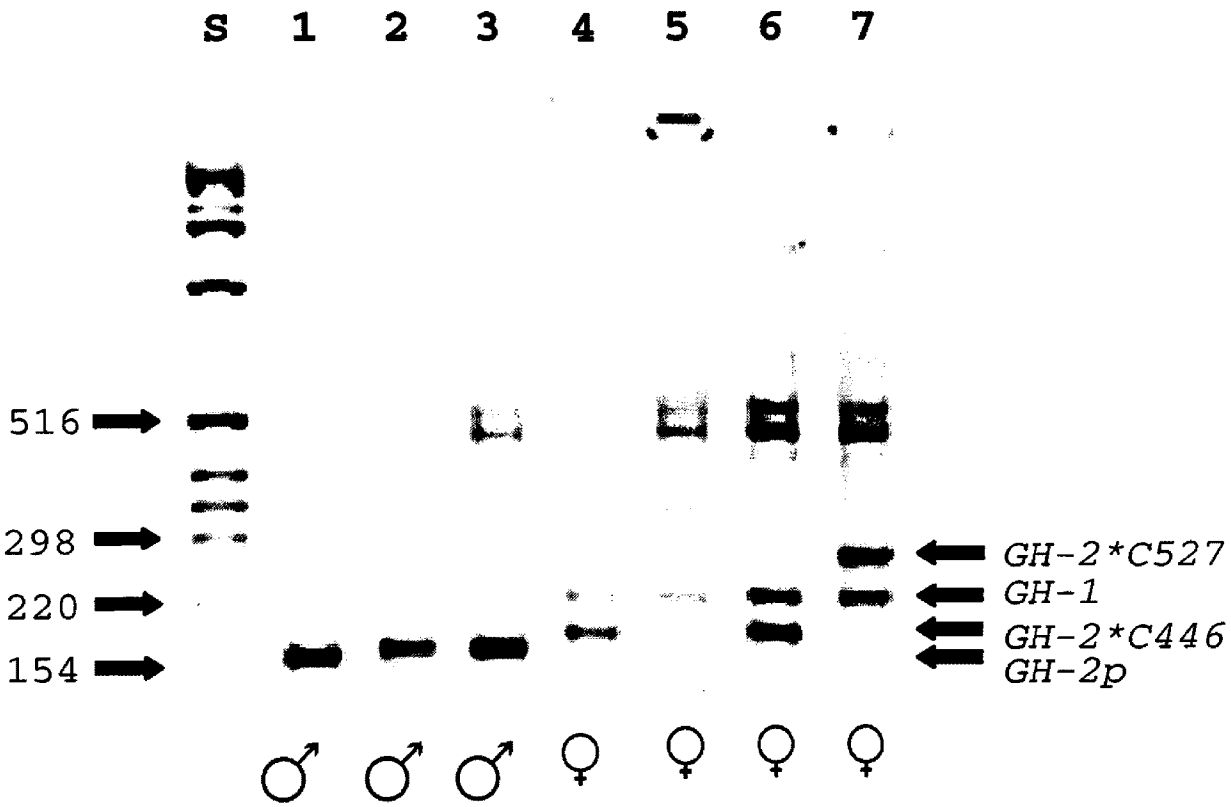


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**Fig. 4.** **Aligned nucleotide sequence of intron C from *GH-2* and *GH-2p*. Unknown sequence is represented by ?; dashes represent gaps introduced to produce optimal sequence alignment.**

```
Atlantic GH-2       GTAAGTTACCTGGCTGAGACAA-TCCTCCATGATGCTAGATTCCAAAA   47
Brown GH-2          .........-...........A.............AT......G...   47
Rainbow GH-2        ......................-.............AC........C.   47
Cutthroat GH-2      ......................-.............AC........C.   47
Chinook GH-2        ......................-.............ACA.......C.   47
Coho GH-2           ......................-.............ACA.......C.   47
Chum GH-2           ......................-.............ACA.......C.   47
Pink GH-2           ......................-.............ACA.......C.   47
Sockeye GH-2        ..........G...........-.............ACA.......C.   47
Coho GH-2p          ..............G.......-...G.-.C.....AC..........   46
Pink GH-2p          ??????????????????????????????????????????????
Chinook GH-2p       ..............G.......-...G.-.C.....AC..........   46
Chum GH-2p          ----------------------------------------------

Atlantic GH-2       TAAATAATAGGGCATCTCAATTTGAACAA-TCGATACAACTTAGTCAT   94
Brown GH-2          ............................-.................   94
Rainbow GH-2        .G..........................-.................   94
Cutthroat GH-2      .G.......T..................-.................   94
Chinook GH-2        .G.........................G..AT..GTTA..G.G..A   95
Coho GH-2           .G.........................G..AT..GTTA..G.G..A   95
Chum GH-2           .G..........................-.................   94
Pink GH-2           .G..........................-.................   94
Sockeye GH-2        .G..................G.......-.................   94
Coho GH-2p          .T........................T..-.................   93
Pink GH-2p          ??????????????????????????????????????????????
Chinook GH-2p       .T..........G...........G.T..-.................   93
Chum GH-2p          ----------------------------------------------

Atlantic GH-2       T-AGTTATTGGGCAAGCAGATCCC-GATTGTGTAAACTCCATGGGTAA  140
Brown GH-2          .-.......................C.....................  141
Rainbow GH-2        .-.......................C......C..............  141
Cutthroat GH-2      .-.......................C......C..............  141
Chinook GH-2        GC..--..C---.---.----.---......C..............  128
Coho GH-2           GC..--..C---.---.----.---......C..............  128
Chum GH-2           .-.......................C......C.........C.....  141
Pink GH-2           .-.......................C......C..............  141
Sockeye GH-2        .-.......................C......C..............  141
Coho GH-2p          .-.......................CA.....C..............  140
Pink GH-2p          ??????????????????????????????????????????????
Chinook GH-2p       .-.......................C......C..............  140
Chum GH-2p          ------------------------------.TCC.............   18
```

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```
Atlantic GH-2      ATATATACTGTAGATTAGCAGAGCCAGCATCATGCATGGTGGAAATTA  188
Brown GH-2         ............G...................C.............  189
Rainbow GH-2       -.....--.......A......A...........T.............  186
Cutthroat GH-2     ..............A......A..........................  189
Chinook GH-2       ..............A......A........T.................  176
Coho GH-2          ..............A......A..........................  176
Chum GH-2          ..............A..A...A..........................  189
Pink GH-2          ..............A..A...A..........................  189
Sockeye GH-2       ..............A..A...A................A.........  189
Coho GH-2p         ..............AA......A..........................  188
Pink GH-2p         ??????????????????????????????????????????????
Chinook GH-2p      G............GAA......A..........................  188
Chum GH-2p         ......C.......AA......A..........................   66

Atlantic GH-2      AATCTAGCCATGACAAGGAGTTTTAAATTGTACACTTAAAA-------  229
Brown GH-2         ..........................................-------  230
Rainbow GH-2       ..............G...........................-------  227
Cutthroat GH-2     ..............G...........................-------  230
Chinook GH-2       ..............G.A.........................-------  217
Coho GH-2          ..............G.A.........................-------  217
Chum GH-2          ..............G............................AAATCAA  237
Pink GH-2          ..............G............................A------  231
Sockeye GH-2       ..............G...........................-------  230
Coho GH-2p         ............T.G...........................-------  229
Pink GH-2p         ??????????????????????????????????????????????
Chinook GH-2p      ............T.G...........................-------  229
Chum GH-2p         ............T.G..........................---------  105

Atlantic GH-2      ---------------------------------------------------  229
Brown GH-2         ---------------------------------------------------  230
Rainbow GH-2       ---------------------------------------------------  227
Cutthroat GH-2     ---------------------------------------------------  230
Chinook GH-2       ---------------------------------------------------  217
Coho GH-2          ---------------------------------------------------  217
Chum GH-2          ATCAAATAAAAAAATGTAATAAAAAAAAATAACAAAATAAAATAAATT  285
Pink GH-2          ---------------------------------------------------  231
Sockeye GH-2       ---------------------------------------------------  230
Coho GH-2p         ---------------------------------------------------  229
Pink GH-2p         ??????????????????????????????????????????????
Chinook GH-2p      ---------------------------------------------------  229
Chum GH-2p         ---------------------------------------------------  105
```

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Atlantic GH-2      --------TC-AGCAGTAAAATGTTGCTATACCTCAGTGCTTTCAACT  268
Brown GH-2         --------..-G.............................C.......  269
Rainbow GH-2       --------..-G.........AA.................C.....--  264
Cutthroat GH-2     --------..-G.........AA.................C.....--  267
Chinook GH-2       --------..A.............................C.......  257
Coho GH-2          --------..A-A...........................C.......  256
Chum GH-2          TAAAAAAA.T-G....G.......................C.....--  330
Pink GH-2          --------..-G....G................A.......C.....--  268
Sockeye GH-2       --------..-G....G.......................C.....--  267
Coho GH-2p         --------..-G.............................C.......  268
Pink GH-2p         ??????????????????????????????????????????????
Chinook GH-2p      --------..AG............G.............C.....T.  269
Chum GH-2p         --------.T-G............T.....-.......C.....T.  143

Atlantic GH-2      AAGGTAGGT-AAAACAACCACATATCACAGTCCTTGTAAGTAAAAC-C  314
Brown GH-2         .........-.....................................-.  315
Rainbow GH-2       .----.----.---............T.................C.  301
Cutthroat GH-2     .----.----.---............T.................C.  304
Chinook GH-2       .........A.....................................C.  305
Coho GH-2          ......-..G.......T.............................C.  303
Chum GH-2          .----.----.---.........T...T.................C.  367
Pink GH-2          .----.----.---.........T...T....A............C.  305
Sockeye GH-2       .----.----.---.........G...T.................C.  304
Coho GH-2p         .......T.-............C.C..T..G.....G.........T.  315
Pink GH-2p         ??????????????????????????????????????????????
Chinook GH-2p      .......T.-.........?..C.C..?..C..............T.  316
Chum GH-2p         .......T.-............C.C..T..............-.T.  189

Atlantic GH-2      -ATCACTCTCTAA-TCGGCGATTTCTCTACGTCTACATTCTCCAGCCA  360
Brown GH-2         -............-.....................................  361
Rainbow GH-2       -............A......G.............................  348
Cutthroat GH-2     -............-......G.............................  350
Chinook GH-2       -............-......G.........................A.  351
Coho GH-2          -............-......G.........................A.  349
Chum GH-2          -............-......G.........................A.  413
Pink GH-2          -............-.T....G...................G....A.  351
Sockeye GH-2       -............-......G.........................A.  350
Coho GH-2p         -............-......GG............................  361
Pink GH-2p         ??????????????????????????????????????.......    7
Chinook GH-2p      -............-......G.........A.............C...  362
Chum GH-2p         T.-....A.....T.??????????????????????????????  203
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Atlantic GH-2       TGTATCATGTAAATGATATGGCATCTCAAGCTGTACAAT-ACAACTCA   407
Brown GH-2          ........................................-........   408
Rainbow GH-2        .......................................T...T....   396
Cutthroat GH-2      ........................................T........   398
Chinook GH-2        ........................................T........   399
Coho GH-2           ...-.--.--.--..-.--.....................T........   387
Chum GH-2           ....................A...................T........   461
Pink GH-2           ........................................T........   399
Sockeye GH-2        ...G....................................T........   398
Coho GH-2p          ....................T...................-....T...   408
Pink GH-2p          G...................T...................-....TG..    54
Chinook GH-2p       ............G.......T....C...C..........-........   409
Chum GH-2p          ????????????????????????????????????????????????   203

Atlantic GH-2       ACTTCATTTTCTAATAATCTGTGGTTTCTCTGCATCTACACACACAG    454
Brown GH-2          ........................-.......A..............    454
Rainbow GH-2        ................................A..............    443
Cutthroat GH-2      ................................A..............    445
Chinook GH-2        ..............C.................A..............    446
Coho GH-2           ..............C.................A..............    434
Chum GH-2           .........-......................A..............    507
Pink GH-2           ................................A..............    446
Sockeye GH-2        ................................A..............    445
Coho GH-2p          ........................T.......A..............    455
Pink GH-2p          ................................A..--------.....     93
Chinook GH-2p       ................................A..............    456
Chum GH-2p          ????????????????????????????????????????????    203
```

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**Fig. 5.** Tree generated using the method of Fitch parsimony from intron C nucleotide data of *GH-2* and *GH-2p*. The tree was rooted with the Atlantic and brown clade. Bootstrap values represent the percentage of 1000 replicates that a node was supported.

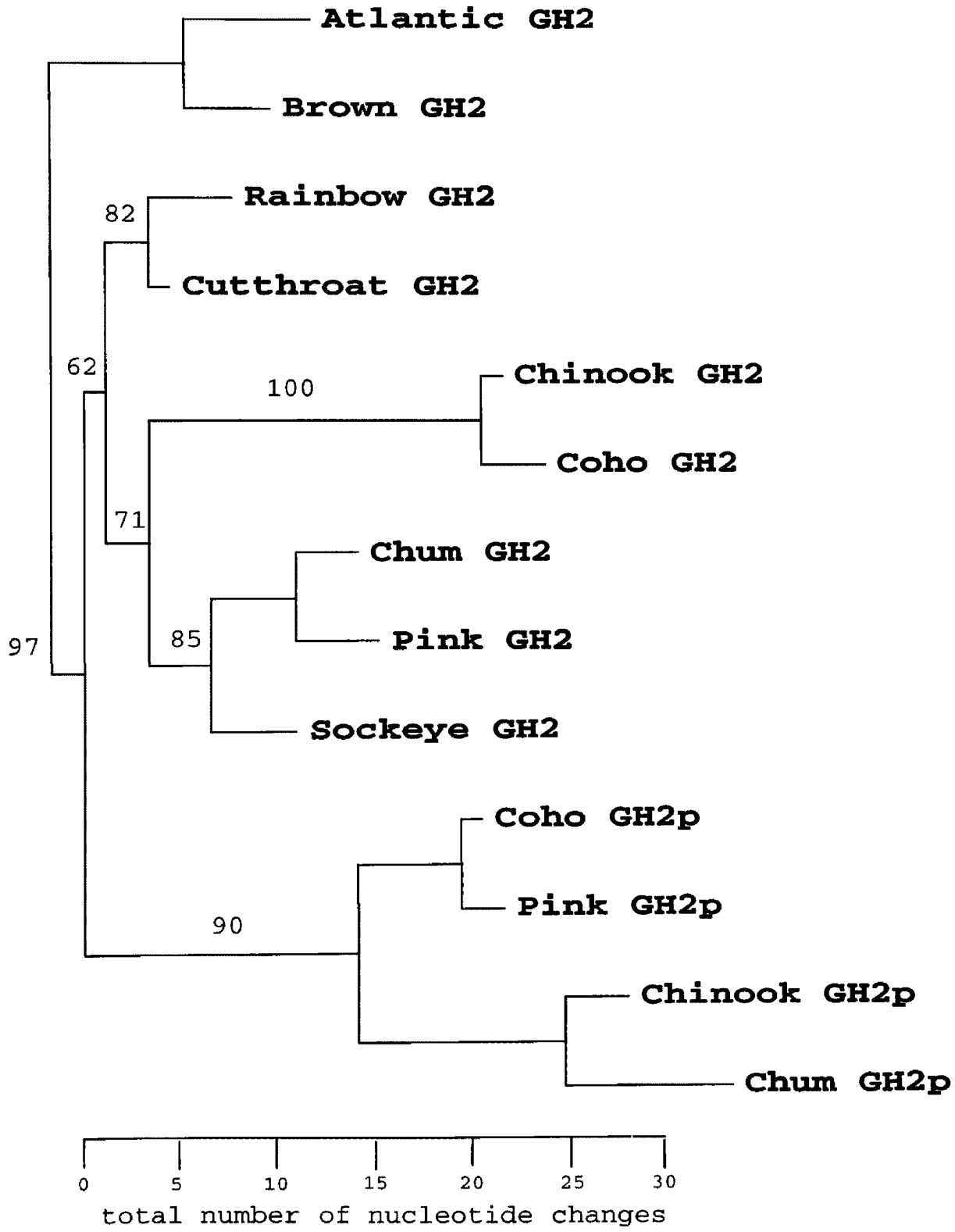


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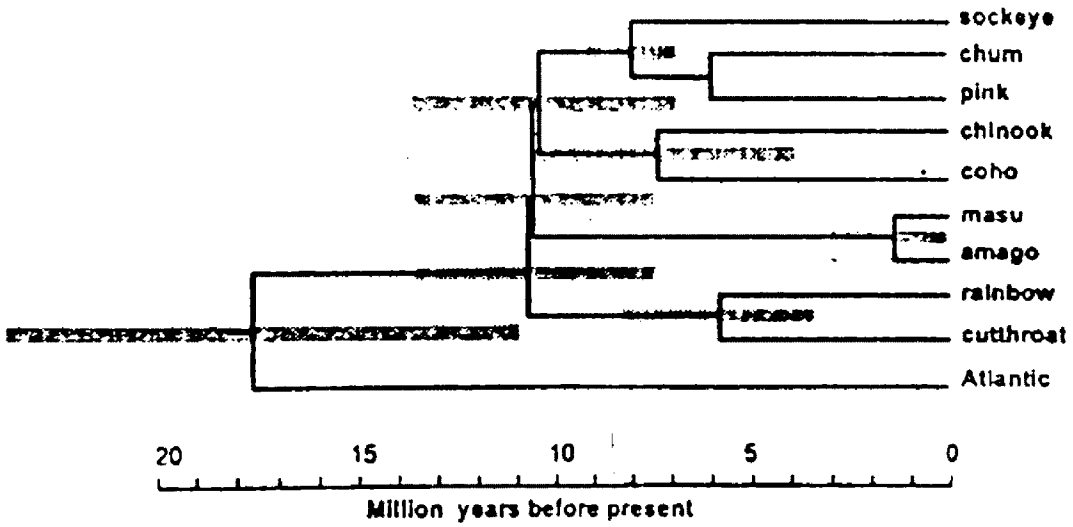
**Fig. 6.** The evolution of *Oncorhynchus* based on the inferred total evidence phylogeny. Shaded bars represent the range between time estimates based on *ND3* and *GH2* sequence divergence. In all cases, the *ND3* time estimate defines the low, or right-most end of the range. To prevent negative branch lengths between internal nodes, the mean time estimates for the (from left to right) second through fourth nodes for *GH2* and second and third nodes for *ND3* were used. The distance between these nodes was exaggerated to emphasize the inferred branching order.

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# The Search for a Sex-Linked Marker in Pink Salmon (*Oncorhynchus gorbuscha*) using Bulked Segregant Analysis

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**ABSTRACT**

The objective of this study was to identify loci closely linked to the sex determining region in pink salmon (*Oncorhynchus gorbuscha*). A marker present within the pseudoautosomal region of the Y-chromosome might also be present on the X-chromosome making it possible to identify the X-chromosome linkage group. Pools of DNA from males, and pools of DNA from females from the same family were created using bulked segregant analysis. Seventy-four amplified fragment length polymorphism (AFLP) primers, and 18 paired interspersed nuclear elements (PINE) primers were used to amplify random, multilocus DNA fragments. A total of ~2400 bands (loci) were examined without detecting a sex-specific marker. The failure to detect a sex-linked marker in this study may be due to the size and complexity of the sex-determining region (*SEX*) on the Y-chromosome.

**INTRODUCTION**



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Currently, a map of the pink salmon (*Oncorhynchus gorbuscha*) genome is being constructed (Allendorf et al. 1997c, Allendorf et al. 1998, Spruell et al. 1999). This map is based on a single female (95-103), and her gynogenetic haploid and diploid progeny. It would be useful to be able to identify the X-linkage group on the map. One way of doing this would be to detect a locus that is closely linked to the sex determining region,(*SEX*) on the Y-chromosome, but located within the pseudoautosomal region such that the locus is also present on the X-chromosome in some individuals.

Finding a marker on the Y-chromosome (linked to the sex determining region) in pink salmon requires a technique that will be able to effectively target that particular region of the genome. Classically, to detect linkage (i.e. between a genomic region of interest and other markers) requires a large number of individuals and is often a labor intensive process (Wang and Paterson 1994). In contrast, a method called "bulked segregant analysis" has been developed to detect genetic markers linked to a target gene or region of the genome that is much less labor intensive than classic linkage studies (Michelmore et al. 1991, Ronin et al. 1996). In bulked segregant analysis, DNA from individuals that segregate for a trait of interest are pooled together (or bulked), such that the target region is homogeneous within pools, but differs between the pools. These pools of DNA

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can then be screened with random genetic markers to detect a marker that differs between the two pools and is thus linked to the genomic region of interest.

Pooling DNA has been shown to be an extremely cost effective and time-saving technique to detect markers linked to a particular genomic region (Giovannoni et al. 1991, Michelmore et al. 1991). Bulked segregant analysis may cut down the number of PCR runs needed to detect a marker linked to a target region by up to 90-97% (Ronin et al. 1996). Bulked segregant analysis can pool DNA from any individuals in a segregating population (e.g. full-sibs, back crossed individuals), and can be used to target specific genes, or chromosomal regions of interest (Giovanonni et al. 1991).

Theoretically, bulked segregant analysis can be used to detect a Y-linked marker in pink salmon where the sex determining region (*SEX*) is the genomic region of interest. Pooling DNA from males and females generated from a single pair mating will create two pools of DNA that differ for *SEX* and the surrounding pseudoautosomal region of the Y-chromosome, yet are genetically similar at all loci unlinked to the target region. However, in order for bulked segregant analysis to be an effective method, it is necessary that a large number of polymorphic loci are screened on the two pools of DNA.

Since the introduction of this method independently by Michelmore et al. 1991 and Giovannoni et al. 1991, randomly

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amplified polymorphic DNAs (RAPDs) and restriction fragment length polymorphisms (RFLPs) have most commonly been used with bulked segregant analysis. Although RFLPs and RAPDs can be useful as molecular markers and in mapping studies, they also have associated problems. RFLPs are often time consuming and laborious, and is the reason RAPDs have often been used solely in bulked segregant analysis (Chalmers et al. 1993, Paran and Michelmore 1993, Chagué et al. 1996). RAPDs generate several DNA fragments, potentially amplify throughout the entire genome, and have been used widely to asses genetic variation. However, in an evaluation of RAPDs in red deer, wild boar, and fruit fly, the reproducibility of banding patterns in the same individual was poor, and the percentage of repeatable RAPD bands ranged from 23-36% (Pérez et al. 1998).

In recent years, new types of molecular markers that amplify multiple loci simultaneously have been developed. Amplified fragment length polymorphisms (AFLPs), take advantage of random priming throughout the genome, but with a degree of specificity. The AFLP method uses the polymerase chain reaction (PCR) to amplify restriction fragments generated by digesting the genome using two restriction enzymes. Special adapter sequences are then ligated to the sticky ends created by the restriction enzymes and PCR is performed using primers specific to the adapter sequence, the restriction site, and an additional

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base in order to reduce the number of genomic DNA fragments originally generated by the restriction enzymes. A second round of PCR is performed using primers specific to the adapters, restriction site, the base used during the first round of PCR, and finally, two additional bases to further reduce the number of fragments so that they can be visualized by gel electrophoresis. The AFLP procedure is able to use multiple primer combinations during the second round of PCR; and an entirely new array of primer combinations is possible by choosing a different original selective base during the first round of PCR.

AFLP primers have proven extremely useful in plant mapping studies (Thomas et al. 1995, Vos et al. 1995) and are highly polymorphic and reliably reproducible in comparison to RAPDs. Reproducibility is high because the annealing temperatures used during PCR are much higher (~60°C) compared with temperatures used for RAPD primers. In a preliminary survey of RAPD and AFLP markers in pink salmon (*Oncorhynchus gorbuscha*), RAPDs produced an average of 2-8 bands per primer, while each AFLP primer pair amplified at least 30 bands, with an average of 7.5 being polymorphic (Allendorf et al. 1997c).

Another class of variable molecular markers takes advantage of short interspersed nuclear elements (SINEs) found throughout the genome. SINEs are characterized by an internal POL III promoter, an A-rich 3' end, flanking direct

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repeats, and may be related to 7SL RNA and tRNA (Okada 1991, Li 1997). SINEs have been found in several mammal species, and various repetitive DNA elements have been discovered throughout the genome of salmonid fishes. Kido et al. (1991) documented the presence of two such elements, *Hpa*I and *Sma*I in pink salmon. Spruell and Thorgaard (1996) subsequently reported the presence of the 5' end of a third element, *Fok*I, in pink salmon. Goodier and Davidson (1994) confirmed that salmonids also contain the transposon Tc1, a member of another class of repetitive elements. Finally, Bois et al. 1998 documented the presence of a family of VNTR repeats (named 33.6+2, in the family of Jeffrey's repeats) in salmonids.

DNA sequences similar to salmonid-specific SINEs and the transposon Tc1, have been used as primers to generate multiple DNA fragments from a single PCR reaction (Spruell et al. 1999). This technique has been called PINEs (paired interspersed nuclear elements). Primers that match one end of the element are oriented such that they initiate DNA synthesis from the end of the element, progressing into the surrounding genomic DNA. A single primer or combinations of primers may be used to generate multilocus patterns.

Pooling DNA from males and females from a single-pair mating, creates two pools of DNA that differ only in their sex chromosome composition (Fig. 1). A polymorphism detected in the male pool that is absent in the female pool

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could indicate a marker that is on the Y-chromosome, and presumably linked to the sex determining region (Fig. 2). It may also be possible to detect a marker specific to the X-chromosome. If a marker is present in the female pool and absent in the male pool, this could indicate a marker specific to the X-chromosome from the male parent (chromosome Xm, Fig. 1). Using AFLP and PINE markers will greatly increase the chances of finding a sex-linked marker due to the greater number of polymorphisms produced per unit effort. I report here on the use of AFLP and PINE markers to find a sex-linked marker in pink salmon using bulked segregant analysis.

## MATERIALS and METHODS

### Pink Salmon Families and DNA Extraction

Two, full-sibling families were used in this study. Pink salmon families from a controlled, single-pair mating were generated at the Armin F. Koering Hatchery in Prince William Sound and at the Genetics Lab facilities of the Alaska Department of Fish and Game in Alaska. The first family, 95-14 was generated from 2, even year sexually mature fish collected in 1996, and represents an even year run sample. The second family, 96-7B was generated from 2, odd-year sexually mature fish, and represent an odd-year run sample.

Embryos were incubated until just prior to hatching

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when they were collected and preserved in ethanol. Eighty-three embryos from family 95-14, and 58 embryos from family 96-7B were collected. DNA was isolated from the embryos after separation from the yolk sac using the Purgene(™) DNA isolation kit (Gentra Systems Inc.) The concentration of DNA was determined using a scanning spectrofluorometer.

**Confirmation of Families**

It was important to test that the individuals chosen for analysis belonged to the correct family in case embryos from another family mistakenly ended up in the wrong rearing trays during hatchery incubations. Confirmation of family identity of 58 embryos from family 96-7B was performed by DNA fingerprinting analysis using primers matching the *Sma*I SINE and *Tc*1 transposon (Greene and Seeb 1997).

Family identity of embryos from family 95-A14 was tested using two microsatellite loci and one DNA fingerprint using primers matching the *Fok*1 SINE and the *Tc*1 transposon. Microsatellite analysis was performed in 10 μl PCR mixtures that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4.0 mM $MgCl_2$, all four dNTPs (each at 0.2 mM), each fluorescently labeled primer at 0.45 *p*M, 0.5 U *Taq* DNA polymerase (Perkin-Elmer/Cetus), and 20 ng of purified genomic DNA. Primers and annealing temperatures are as follows: μSat60, 55°C (Estoup et al. 1993); and Ots1, 55°C (Hedgecock D, personal communication). PCR products were electrophoresed on a 7% denaturing polyacrylamide gel, and visualized with a Hitachi

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FMBIO-100 fluorescent imager. The individuals tested from family A14 showed Mendelian inheritance and allelic patterns consistent with the parental genotypes at both microsatellite loci used for screening (Table 1).

PCR amplification of DNA fragments flanked by the *Fok*1 SINE and the *Tc*1 transposon were conducted in a total reaction volume of 10 $\mu$l. PCR mixtures contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5.0 mM $MgCl_2$, all four dNTPs (each at 0.2 mM), each fluorescently labeled primer at 0.4 *p*M, 0.1 unit of *Taq* DNA polymerase (Stoffel fragment; Perkin-Elmer/Cetus), and 20 ng of DNA. PCR products were electrophoresed on a 4% denaturing polyacrylamide gel, and visualized with a Hitachi FMBIO-100 fluorescent imager. Ninety-three individuals from family 95-A14 demonstrated banding patterns consistent with the parents used to create the family.

**Sexing Embryos**

DNA from all embryos from family 95-A14, and family 96-7B was amplified using primers designed from chinook salmon that target the growth hormone pseudogene (*GH-2p*; R. H. Devlin personal communication). This Y-linked pseudogene allows the sex of unknown individuals to be determined. PCR was performed in mixtures that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5.8 mM $MgCl_2$, all four dNTPs (each at 0.2 mM), each primer at 0.3 *p*M, and 0.08 unit of *Taq* DNA

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polymerase (Perkin-Elmer/Cetus), and 40 ng of template DNA in a total volume of 15 μl. The PCR profile was 30 cycles of 92°C for 1 min, 51°C for 1 min, and 72°C for 2 min. PCR products were electrophoresed on 2.0% agarose gels in TAE buffer (Ausubel et al. 1989), containing ethidium bromide at 0.5 μg/ml. Males were identified by the presence of a 166 bp fragment that is absent in females (previously discussed, chapter 2). Twenty individuals identified as males, and 20 individuals identified as females from each family were chosen for subsequent DNA analysis.

**DNA Pooling**

The number of individuals to include in a pool is a balance between a pool that is large enough to avoid detecting false positives (unlinked loci that appears polymorphic between the pools) but small enough to maximize the length of chromosome that remains polymorphic between the pools (Giovannoni et al. 1991, Churchill et al. 1993, Ronin et al. 1996). It has been recommended that more than 5 individuals per pool be used (Michelmore et al. 1991) and that 10 individuals is sufficient to avoid most false positives (Wang and Paterson 1994). The size of the sex determining region on the Y-chromosome in pink salmon and other salmonids is unknown. Therefore, using smaller pools of individuals will maximize the chance of detecting markers closely linked to the sex-determining region of the Y-chromosome.

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Individuals to be included in the pools of DNA were chosen based on their sex and the quality of DNA (as measured by quantity with the spectrofluorometer). Two male pools of DNA (M1 and M2), and two female pools of DNA (F1 and F2) were created from family 95-A14 using different individuals in each. One male pool (7B-M) and one female pool (7B-F) were created from family 96-7B. Each pool of DNA was created by combining .5 μg of DNA from each of 10 individuals (Fig. 3).

**AFLP and PINE Analysis of DNA Pools**

Ten ng/μl of the pooled DNA was used in the AFLP restriction/ligation and pre-selective amplification steps according to the Perkin-Elmer/Applied Biosystems AFLP plant mapping protocol with the modifications outlined below. Ten μl selective amplification reactions contained 1.5 μl pre-amplification products as DNA template, 0.5 μl *Eco*RI selective primer, 0.5 μl *Mse*I selective primer, 2.0 mM $MgCl_2$, all four dNTPs (each at 0.1 mM), 2X Amplitaq PCR buffer, and 0.5 U of *Taq* DNA polymerase (Perkin-Elmer/Cetus). The PCR profile for selective amplification was: initial denaturation at 96°C for 2 minutes, followed by a series of 7 cycles with denaturation at 96°C for 1 second, annealing at 65°C for 30 seconds, extension at 72°C for 2 minutes. The annealing temperature was decreased by 1 degree per cycle for 6 cycles; and an additional 30 cycles with an annealing temperature of 59°C for 30 seconds

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followed. Products were electrophoresed on a 7% denaturing polyacrylamide gel and visualized using a Hitachi FMBIO-100 fluorescent imager.

PCR amplification of DNA fragments flanked by interspersed elements (PINEs) was performed using primers described in Table 2. PCR mixtures were performed in a total reaction volume of 10 $\mu$l and contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5.0 mM $MgCl_2$, all four dNTPs (each at 0.2 mM), each fluorescently labeled primer at 0.4 *p*M, 0.1 unit of *Taq* DNA polymerase (Perkin-Elmer/Cetus) and 20 ng of the pooled DNA. PCR products were electrophoresed and visualized in the same way as AFLPs.

Primers that generated putative polymorphisms seen between male and female pools using AFLP and PINE primers were subsequently confirmed by segregation analysis of individuals. These individuals included those used to create the pools of DNA. Deviations from the null hypothesis of 1:1 segregation for males and females were tested by a two-tailed binomial probability test.

**Robustness of Pooling**

Five AFLP primer pair combinations, and 3 PINE combinations were initially tested for priming consistency on both individuals and their respective DNA pools. Pools M1 and F1 from family 95-A14 and the individuals comprising the pools were tested. Testing both AFLP primers, and PINE primers showed that all bands present in the individuals

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were also present in the pooled DNA samples. The test gave an estimate of the robustness of the pooling technique, showing that with both AFLPs and PINEs, a band present in only one of the ten individuals comprising the pool was also present in the pooled DNA sample.

The sensitivity of detection estimate for AFLP and PINE markers is consistent with results observed using RAPD and RFLP markers. Using a single-copy DNA probe in Southern blot analysis, Churchill et al. 1993 was able to detect a rare allele in a mixture as low as 40:1 for RFLP markers. The limit of detection for RAPD markers was around a ratio of 10:1, although band intensity seemed to be band (locus) specific (Michelmore et al. 1991).

**RESULTS**

AFLP primer pairs produced scorable, multi-locus bands in the pooled DNA samples with sizes ranging from 75-400 bp (Fig. 4). Seventy-four AFLP primer pair combinations were screened on the six pools of DNA. DNA fragments flanked by SINEs, the transposon Tc1, and a VNTR repeat 33.6+2 produced scorable, multilocus fragments in the pooled DNA samples. Using two different PINE primers (e.g. HpaI5'/SmaI5', HpaI3'/Tc15') produced around 20 bands, with products ranging in size from 75-450 bp. Using the same PINE primer (e.g. SmaI5') to generate multiple DNA fragments produced fewer bands with products ranging from around 200-500 bp.

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Fifteen PINEs using primers from different elements, and three PINEs using a single primer were screened on the pooled DNA samples.

A total of eight AFLP primer pairs produced potentially sex-specific products in the pooled DNA samples (Table 3). Three AFLP primer combinations produced bands present in the male pools of family 95-A14 (M1 and M2) and absent in both the female pools from this family and the male and female pools from family 96-7B. Four AFLP primer combinations produced bands present in 7B-M and 7B-F from family 7B that were absent from all other pools. One AFLP primer combination produced a product present in the female pools of family 95-A14 (F1 and F2) that was absent from all other pools. No AFLP primer combination produced apparent sex-specific bands in males or females from both families.

Testing the potentially sex-specific primer pairs upon 40 individuals from each family (20 males and 20 females) did not show a statistical deviation from expected 1:1 segregation pattern for males and females (Table 4). The eight AFLP and one PINE primer pair combination tested on the individuals, resulted in the potentially sex-specific band seen in the pooled DNA sample was present in only a few individuals (usually 5 or 6) of both sexes.

One PINE primer combination (Fok1 5'/Tc1 5') produced a 260 bp fragment present in the female pools (F1 and F2) that was absent in all other pools (Table 3). These primer

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combinations were then screened in the 40 individuals from family A14. The 260 bp fragment was present in only 12 females and 8 males and was not sex-linked.

All potential polymorphisms seen between pools that differed by sex were false positives of markers occurring in low frequencies. Based on a sample of 25 AFLP selective primer pairs used in this study, an average of 33.6 bands are produced per primer pair. Therefore, on the order of 2000 bands (loci) were screened with the 74 AFLP selective primer pairs. However, of the total bands screened, around 555 polymorphic bands were probably screened (based on an average of 7.5 polymorphisms produced per primer pair; Allendorf et al. 1997c, personal observation) without detecting a sex-linked marker. For PINEs, an average of 21.8 bands per primer pair are amplified in pink salmon (based on a sample of 10 PINE primer combinations used in this study) suggesting that around 400 PINEs were examined with the 18 PINE primer combinations used. Based on 3 primer pair combinations tested on individuals, an average of 4 polymorphisms are produced per PINE primer pair (personal observation), so around 72 polymorphisms were screened without finding a sex-linked marker.

Both AFLP and PINE primer combinations produced fragments that were specific to either the odd or even year family. However, family differences were not able to be distinguished from odd and even year-class differences in

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this study.

## DISCUSSION

In previous studies, bulked segregant analysis has been a successful method for detecting markers linked to a particular region of the genome. Random markers such as RAPDs and RFLPs have been used successfully with bulked segregant analysis to find markers linked to disease resistance genes in several plants (Michelmore et al. 1991, Ballvora et al. 1995, Borovkova et al. 1995, Meksem et al. 1995). Additionally, bulked segregant analysis has been successful in detecting genetic markers linked to sex determination. One RAPD marker linked to the sex determining region in pistachio was detected after screening 700 RAPD primers (Hormaza et al. 1994). Similarly, one RAPD marker linked to sex determination in the basket willow after screening 380 RAPD primers (Aistrom-Rapaport et al. 1998). Bulked segregant analysis using AFLPs detected nine markers (out of 253 primer pairs screened) linked to the sex locus in *Asparagus officinalis* L. (Reamon-Büttner et al. 1998).

Detecting sex-linked markers in salmonids has been difficult. The sex chromosomes are not highly differentiated from one another cytogenetically, and genetically are thought to be the same except for the sex determining region found on the Y-chromosome. Previously,

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60 RAPD primers that were tested on individual (15 male and 30 female) rainbow trout failed to produce sex-specific markers (Moran et al. 1996). Screening with RAPD primers also failed to detect a male-specific marker in Atlantic salmon (McGowen and Davidson 1998). However, bulked segregant analysis used in conjunction with fluorescent *in situ* hybridization (FISH) was able to identify two Y-linked RAPD markers in rainbow trout (Iturra et al. 1998).

In this study, bulked segregant analysis was used to try to detect a marker located within the pseudoautosomal region of the X and Y chromosomes in pink salmon. In order to have a representative sample of pink salmon, families from both odd and even year classes were used because of the large genetic differences observed between odd and even year classes (Phillips and Kapuscinski 1988). Using microsatellite loci, and a DNA fingerprinting technique using primers with sequences similar to interspersed elements, it was ensured that all embryos used in this study were from the correct family. Embryos from each family were sexed using the Y-linked, growth hormone pseudogene (Chapter 2). While sexually mature pink salmon show great morphological differentiation (U.S. Fish and Wildlife Service 1989), it is impossible to sex embryos and immature fish based on morphology. The ability to amplify a Y-linked growth hormone pseudogene in males (chapter 2) makes it possible to unambiguously identify males and females

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genetically.

The methods of pooling DNA for bulked segregant analysis have been explored in other studies (Wang and Paterson 1994, Churchill et al. 1993). For effective pooling, it is important that DNA from all individuals is represented equally. This was ensured by determining the concentration of DNA using a scanning spectrofluorometer, and adding an equal amount of DNA from each individual to the pool.

A second concern of pooling DNA for use in bulked segregant analysis is the ability to detect a marker linked to the target region. Other studies use recombinant lines that differ for a trait or gene of interest, and are concerned with the pools containing enough recombinant chromosomes for informative mapping information. Similarly, fewer individuals were pooled together in this study to maximize the chance for recombinants within the pseudoautosomal region of the sex chromosomes. In addition, when phenotypes are used to define the trait of interest (instead of using known mapped genomic markers) the size of the targeted region is unknown. Therefore, when the size of the region is unknown and when marker densities are low, smaller pools are desired (Churchill et al. 1993). Ten pink salmon were chosen for use in each pool because it has been determined that ten individuals is the optimal pool size in order to detect closely linked markers (Michelmore et al.

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1991, Wang and Paterson 1994)

A third concern is the ability to confirm that a marker detected in the pools of DNA is actually linked to the region of interest, and is not a false positive. This is done by testing the potential marker on individuals and testing for segregation patterns that differ from an expected 1:1 of males and females for loci that are not sex-linked. False positives (i.e. a rare allele unlinked to the target region present in one of the pools), are more of a problem when pools of DNA are composed of fewer individuals. In this study, the ability to detect a linked marker was more of a concern than the risk of false positives since any marker that showed a polymorphism between the male and female pools was subsequently screened in individuals from the family.

For bulked segregant analysis to be an effective method of detecting markers linked to a region of interest, it is important to use genetic markers that are randomly distributed throughout the genome. Traditionally, RFLP and RAPD markers have been often used for these types of studies (Michelmore et al. 1991, Ballvora et al. 1995). However, RFLP analysis is often time consuming, and RAPD fragments may not be reliably reproducible (Pérez et al. 1998). In addition, limited polymorphisms were detected in pink salmon haploid families (Allendorf et al. 1997).

Recently, two new methods to amplify multilocus

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fragments from a single PCR have been developed. AFLP and PINE analysis are advantageous due to the large number of polymorphisms produced per primer pair and the high reproducibility of the fragments. AFLP and PINE markers have been shown to be fairly randomly distributed along the length of chromosomes in pink salmon (Allendorf et al. 1998), making these markers useful for targeting the sex-determining region.

Results from other studies demonstrate that bulked segregant analysis used with highly polymorphic markers such as AFLPs is an extremely effective technique for finding genomic markers linked to a genomic region of interest. Using RAPD primers, it was estimated that all segregating markers within 10% recombination of the target region should be detected, and markers within 30% recombination would often be detected; the limit of detection seemed to be approximately 25 cM (Michelmore et al. 1991).

Unfortunately, bulked segregant analysis of pink salmon males and females using AFLP and PINE techniques failed to detect a sex-linked marker in this study. It is possible that a greater number of primer combinations were needed to be screened on the pooled samples of male and female DNA from pink salmon to detect a marker closely linked to *SEX* in this species. The failure to detect a sex-linked marker with this technique could be a factor of the size of the targeted region. It is unknown how much of the Y-chromosome

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the sex determining region occupies in salmonids. Since it is difficult if not impossible to differentiate the sex chromosomes in most species of salmonids, it is likely that the sex-determining, non-recombining region comprises a small physical portion of the Y-chromosome. The haploid pink salmon genome is estimated to be 6744 cM (Allendorf et al. 1998). However, this size is based on a female, and is expected to be larger than for males since males show a reduced rate of recombination relative to females (May and Johnson 1990). A conservative estimate of the genome size of the male pink salmon based on a male rainbow trout (around 2627 cM; Young et al. 1998), and reduced recombination in males might put the male pink salmon genome size between 3000 and 6000 cM. Given this, and the ability to reliably detect markers linked to a targeted region up to around 25 cM, the targeted region was between 120 and 240 cM on either side of the sex determining region, which is a fairly small region to target.

The sex determining region in salmon may also be fairly complex. Of the relatively few sex-linked loci known in salmonids, the locus *GH-2p* is Y-linked in chinook salmon, coho salmon, chum salmon, and pink salmon. However, cosmids developed from the surrounding DNA of *GH-2p* in chinook failed to detect Y-linked DNA in the other three species (Devlin et al. in preparation). Unlike the *SRY* gene (sex-determining region Y) in mammals, no widely conserved sex-

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specific marker has been detected in non-mammals (Griffiths and Tiwari 1993). In salmonids, the lack of conserved, sex-linked markers between closely related species (see Chp. 1) indicates the evolution of sex chromosomes has occurred in different lineages and at different times. Additionally, the few-sex linked loci known in salmonids, and the failure to detect a random sex-linked marker using bulked segregant analysis in pink salmon, suggests the sex determining region is extremely small, and the X and Y chromosomes are mostly homologous with one another.

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**Table 1.** Observed and expected (parentheses) genotypes of two microsatellite loci in 83 individuals from family 95-A14. The parental genotypes are given under MP and FP (for "male parent" and "female parent").

| | | | Progeny Genotypes | | | | |
|---|---|---|---|---|---|---|---|
| **LOCUS** | **MP** | **FP** | *110/110* | *110/116* | | | $X^2$ |
| *uSAT60* | *110/110* | *110/116* | 45 | 38 | | | 0.59 |
| | | | (41.5) | (41.5) | | | |
| **LOCUS** | **MP** | **FP** | *222/224* | *222/230* | *224/224* | *224/230* | $X^2$ |
| *OTS1* | *222/224* | *224/230* | 13 | 22 | 32 | 16 | 3.40 |
| | | | (20.8) | (20.8) | (20.8) | (20.8) | |

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**Table 2.** Primer Sequences used for paired interspersed nuclear elements (PINE) PCR and reference.

| Primer Name | Sequence | Reference |
|---|---|---|
| HpaI 5' | AACCACTAGGCTACCCTGCC | Kido et al. 1991 |
| HpaI 3' | ACAGGCAGTTAACCCACTGTTCC | Kido et al. 1991 |
| FokI 5' | CTACCAACTGAGCCACACG | Kido et al. 1991 |
| SmaI 5' | AACTGAGCTACAGAAGGACC | Kido et al. 1991 |
| Tc1 5' | GTATGTAAACTTCTGACCCACTGG | Greene and Seeb 1997 |
| 33.6+2 | GGAGGAGGGCTGGAGGAGGGCGC | Bois et al. 1998 |

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**Table 3.** Pattern of potentially sex-specific fragments observed in the pooled DNA samples using eight AFLP and one PINE primer combinations. An "X" indicates the presence of a particular fragment.

| Primer Combination | Fragment Size (bp) | Family 95-14 | | | | Family 96-7B | |
|---|---|---|---|---|---|---|---|
| | | M1 | M2 | F1 | F2 | 7B-M | 7B-F |
| ACG/CTA | 420 | X | X | | | | |
| ACG/CTG | 380 | X | X | | | | |
| AAC/CAG | 180 | X | X | | | | |
| AGG/CTG | 230 | | | X | X | | |
| ACG/CTT | 280 | | | | | X | |
| AAG/CGA | 140 | | | | | X | |
| AGG/CAT | 120 | | | | | X | |
| AGG/CGA | 260 | | | | | X | |
| Fok1 5'/Tc1 5' | 250 | | | X | X | | |

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**Table 4.** Segregation analysis of 20 males and 20 females from each family tested using eight AFLP primer combinations, and one PINE that produced potentially sex-specific bands seen in analysis of pooled DNA samples. P-values represent a test of deviation from expected 1:1 segregation of males to females if the locus is not sex-linked.

| Primer Combination | Fragment Size (bp) | Family 95-14 M | Family 95-14 F | Family 96-7B M | Family 96-7B F | p-val |
|---|---|---|---|---|---|---|
| ACG/CTA | 420 | 4 | 3 | | | 1.00 |
| ACG/CTG | 380 | 6 | 4 | | | 0.75 |
| AAC/CAG | 180 | 7 | 5 | | | 0.77 |
| AGG/CTG | 230 | 3 | 5 | | | 0.15 |
| ACG/CTT | 280 | 7 | 6 | | | 1.00 |
| AAG/CGA | 140 | | | 1 | 3 | 0.63 |
| AGG/CAT | 120 | | | 5 | 8 | 0.58 |
| AGG/CGA | 260 | | | 6 | 7 | 1.00 |
| FokI 5'/TcI 5' | 250 | 5 | 6 | | | 1.00 |

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**Fig. 1.** Punnett square showing the inheritance of sex chromosomes in progeny used in this study.

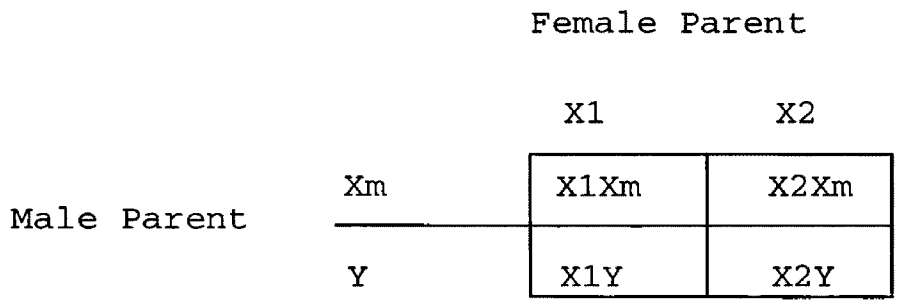


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**Fig. 2.** Diagram showing the X and Y chromosomes in pink salmon. The circles represent the centromere, the rectangle represents the sex determining region (*SEX*). The arrows represent the region of the Y-chromosome being targeted by bulked segregant analysis.

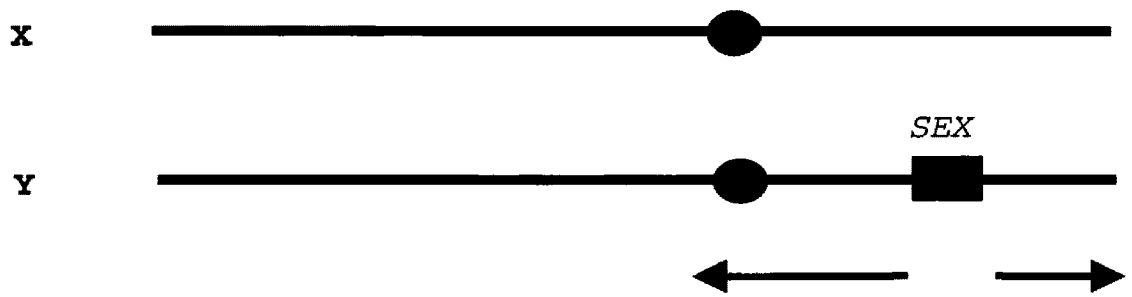


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**Fig. 3.** Diagram showing the pooling procedure. Pools were created using 0.5 ug of DNA from each individual. Males and females were determined by previous screening for the Y-linked *GH-2p*.

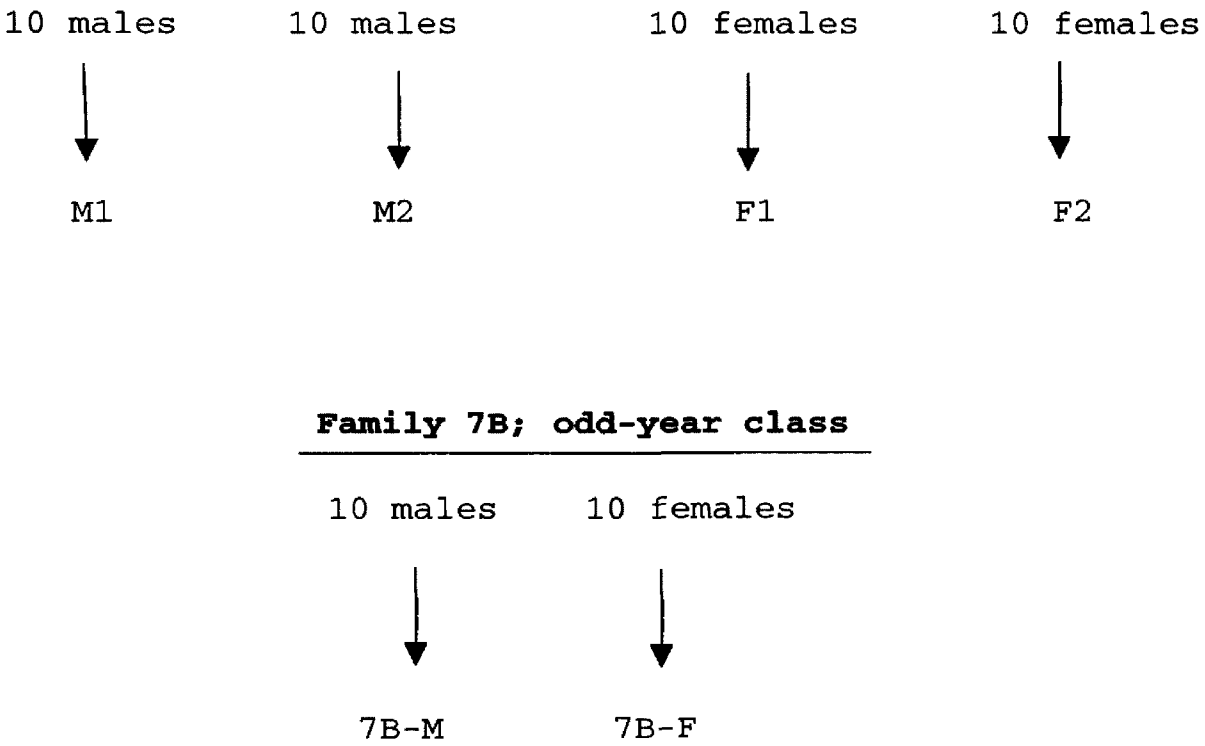


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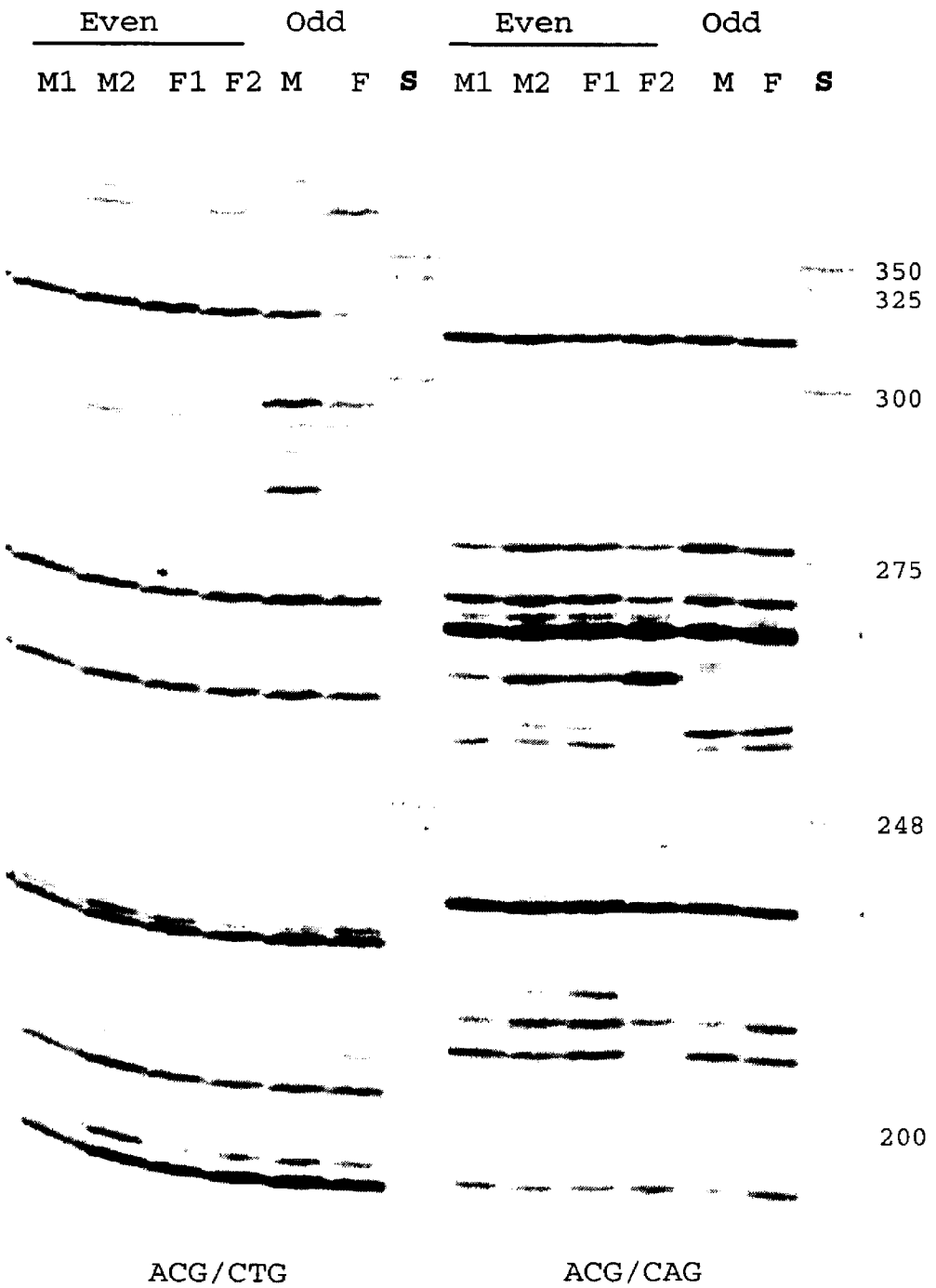
**Fig. 4.** Polyacrylamide gel showing AFLP PCR products from two AFLP primer combinations (ACG/CTG and ACG/CAG) on pooled DNA. The first four lanes of each primer combination are pooled DNA from males and females from the even-year family A14. The last two lanes of each primer combination are the male and female pool from the odd-year family 7B. Lanes with standards are denoted by an "S".

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